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# MICROBIOLOGY

*(Mikrobiologiya)*

Dr. J. R. Porter, Advisory Editor



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ERRATA
Vol. 29, No. 5

On p. 540 of the article, "Ways of increasing the amylolytic activity of industrial enzyme preparations produced by molds" by E. Ya. Kalashnikov et al.:

Change the fifth line of the heading to read:
Translated from Mikrobiologiya, Vol. 29, No. 5, pp. 749–756, September-October, 1960.

Change the footnote on the same page to read:
*SC, i.e., saccharifying capacity, is determined on enzyme preparations of Aspergillus oryzae in accordance with the method set forth in RTU Ukr. SSR 45–58.

# PRODUCTS OF THE FIXATION OF LABELED CARBON DIOXIDE BY HYDROGEN BACTERIA DURING THE COURSE OF CHEMOSYNTHESIS

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Translated from Mikrobiologiya, Vol. 29, No. 6, pp. 795–801, November-December, 1960
Original article submitted February 16, 1960

The clarification of the pathway of carbon during chemosynthesis is of great interest from the point of view of evolutionary and comparative biochemistry. At the present time the similarity of the basic pathways of metabolism in various organisms is established. At the same time, Academician Shaposhnikov is developing a point of view concerning the possibility of the existence of profound differences in the chemism of individual metabolic processes in different organisms, since the conditions of their growth and development as well as the end products of their vital activity are extremely different (Shaposhnikov, 1957).

It can be assumed that the position presented above concerning differences in the chemism of metabolic reactions will prove to be even more clearly pronounced when comparing such different types of organisms as higher plants and bacteria, and will also be extended to the chemism of the assimilation of carbon dioxide by them. The possibility of finding substantial differences in the composition of the product by photosynthesis in higher plants and chemosynthesis in bacteria has therefore not been excluded.

Hydrogen bacteria were used as the material in our investigations; these are facultative autotrophs which, in the absence of organic material, are capable of growing and reproducing by utilizing the energy of oxidation of hydrogen by molecular oxygen in the presence of carbon dioxide as the sole carbon source.

The culture of hydrogen bacteria was isolated from sand taken from the littoral zone of the Moscow River in the neighborhood of the Lenin Mountains according to the method described by Belyaeva (1950). The isolation procedure—inoculation of liquid mineral medium of the following composition: $NaHCO_3$—1.0 g, $K_2HPO_4$—0.5 g, $KNO_3$—2.0 g, $MgSO_4$—0.2 g, $FeCl_3$ — traces, in 1 liter of distilled water, in an atmosphere containing 65% $H_2$, 13% $CO_2$, and 22% air; transfer of pellicle formed to petri dishes of warm agar medium of the same composition with mixing at moment of inoculation; four subsequent transfers of agar slants from single colonies.

As the result of the isolation, a culture was obtained consisting of homogeneous rods with rounded ends, measuring about 1 $\mu$ in length. In our experiments the multiplication of the bacterial culture was brought about by various methods. In the first series of experiments, the bacteria were washed off the surface of the agar slant of the last transfer and were planted on mineral agar medium in petri dishes. The culture, growing under autotrophic conditions for 6-7 days, was washed off the surface of the agar with M/30 phosphate buffer at pH 7.7, was centrifuged at 4500 rpm, and was washed by resuspending twice in phosphate buffer with repeated centrifugation. The suspension of bacterial cells in a small volume of phosphate buffer was the initial material in the first of two variants of our experiments. In this variant, sodium bicarbonate, $NaHC^{14}O_3$, dissolved in nutrient medium served as the carbon source, and conditions were created which were very close to the natural physiological conditions of growth of the given microorganism.

In order to insure such conditions, a special apparatus was constructed consisting of two glass dropping funnels arranged one directly above the other (A and B, Fig. 1), communicating with one another by means of a stopcock (1, Fig. 1), and equipped with stopcocks for the intake of gases (3 and 4, Fig. 1). Bacteria suspended in culture medium devoid of sodium bicarbonate were placed in the lower funnel, B. The upper funnel contained the same medium with an increased content of carbon-labeled $NaHC^{14}O_3$. The apparatus was filled with a gas mixture of the composition: 65% $H_2$, 13% $CO_2$, and 22% air, and was placed in an incubator at 30° C. By alternately opening stopcocks 1 and 2, the microbial suspension could be mixed with the labeled soda, and samples could be taken after quite short exposures to $NaHC^{14}O_3$ (3 sec—minimal).

At very short exposures, however, adequate mixing of the bacteria with the isotope solution was somewhat difficult to accomplish; therefore, it was not possible to obtain reproducible quantitative data regarding the rate of fixation of labeled soda. The greatest difficulties when the experiment was set up in this manner

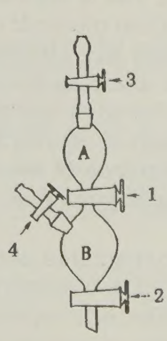


Fig. 1. Apparatus for carrying out experiments on the fixation of $NaHCO_3$ by hydrogen microbes.

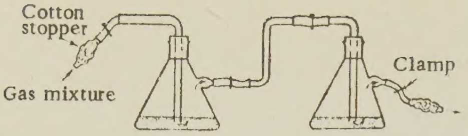


Fig. 2. Apparatus for growing hydrogen microbes in liquid medium.

were connected with the presence in the experimental mixture of a considerable amount of mineral salts — components of the nutrient medium—which interfered greatly with the chromatographic identification of the products of chemosynthesis. This was most markedly pronounced with short exposures of bacteria to labeled soda (3-30 sec), when the total radioactivity of the labeled products formed was low, and large amounts of the original extract had to be spotted on the chromatograms in order to get satisfactory radioautographs.

Attempts to get rid of the salts by using ion exchange resins have so far proved to be unproductive, since among the brands of ion exchange resins at our disposal, not a single one was capable of adsorbing only mineral ions while leaving the ions of organic compounds in solution.

Therefore, a different approach was used for freeing the experimental mixture of an excess of mineral ions.

In this series of experiments the culture of hydrogen bacteria multiplied in a liquid medium containing: $MgSO_4 \cdot 7H_2O$—0.2 g, NaCl—0.2 g, $NaHCO_3$—0.7 g, $KH_2PO_4$—1.0 g, $NH_4Cl$—1.0 g, $FeCl_3$—traces, in 1000 ml of water (Wilson et al., 1953).

The medium was dispensed in Bunsen flasks and a gas mixture consisting of 50% $H_2$, 10% $CO_2$, and 40% air was blown through it (Fig. 2). The same culture as in the first variant was used as inoculum.

On the day that the experiment was set up, a known volume of bacterial suspension was filtered almost to dryness on a bacterial (membrane No. 3) filter. Then, with the aid of a special device in the form of a metal grid on a wooden handle, the filter with the sediment of bacterial culture was rapidly transferred for five minutes to a moist chamber with an atmosphere consisting of 75% $H_2$ and 25% air, floating over mercury, and then into the same kind of chamber containing 3.5% labeled carbon dioxide in air as had been suggested by one of the authors for experiments with green leaves (Doman, 1955, 1958).

This method, which to the present time has not been used for investigating the incorporation of labeled carbon into microbiological material, has a number of advantages in comparison with the first method: 1) The mineral ions of the nutrient medium are eliminated, which is particularly important in studying the early products of chemosynthesis which were of the greatest interest to us; 2) uniform contact of the bacterial mass with $C^{14}O_2$ along the entire surface of the filter is insured; 3) the setting up of the experiment itself is substantially facilitated.

A comparison of the radiochromatograms obtained in the first and second variants of the experiment showed that they were identical. The microbes were killed with cold 96% alcohol. After the suspension was dried under vacuum over dry alkali, the residue was treated with acidified alcohol (10 ml of 1 N HCl per 90 ml of 96% alcohol).

The separation of the labeled products into two fractions—neutral and acid products on the one hand, and compounds containing basic groupings on the other, was accomplished by treating the aqueous solutions with KU-1 (strongly acid) cation-exchange sulforesin under static conditions.

A special check established that this brand of cation exchange resin absorbs practically all amino acids under static conditions, leaving sugars, organic acids, and the phosphoric esters of sugars and glyceric acid in solution. Furthermore, it was established that when sucrose is treated with KU-1 cation exchange resin, it is partially or completely (depending on the ratio of the components used) hydrolyzed to glucose and fructose, and that mineral phosphate splits off from glucose-1-phosphate. The hydrolysis of sucrose by the action of strongly acid sulforesins of other brands has already been noted in the literature (Samuelson, 1955) and there are indications that acidity equivalent to that of a strong solution of sulfuric acid is produced in the pores of this type of cation exchange resin.

The compounds absorbed by the cation exchange resin were eluted with 6 N ammonia solution.

The labeled products were identified by the paper chromatography method, using various solvent systems in combination with radioautography.

It was established that with short exposures 3-30 sec) practically all of the labeled carbon assimilated by the bacteria appeared in the fraction which was soluble in acidified alcohol. With longer exposures the precent of alcohol-soluble products decreased, and after a two-hour exposure of bacterial cells to labeled carbon dioxide, it constituted 70–80% of the radioactivity of the suspension.

Essentially all alcohol-soluble labeled products formed at the exposures indicated above (95.5–97.0%) passed into the aqueous solution.

The table gives data on the distribution among the fractions of the labeled products formed during prolonged chemosynthesis by hydrogen bacteria in nutrient medium containing labeled soda.

It is seen from the table that with prolonged exposures of hydrogen bacteria to $NaHC^{14}O_3$, the fraction containing substances which were not absorbed by KU-1 cation exchange resin constituted 25–30% of the total radioactivity of the aqueous extract, while 44.5–57.8% of the total radioactivity was found in the ammonia eulate from the cation exchange resin.

It should be noted that the reverse ratio of these fractions is obtained when radioactive extracts of leaves of green plants are similarly treated following photosynthesis in an atmosphere of labeled $C^{14}O_2$. Thus for example, according to our data, after five minutes of photosynthesis in $C^{14}O_2$, the labeled sugars, organic acids, and phosphoric esters in barley leaves comprised as much as 66.6% of total radioactivity, while amino acids (eulate of KU-1 cation exchange resin) accounted for only 11% of the total radioactivity of water

Distribution of Radioactivity Among Fractions Following Treatment of an Aqueous Extract of Hydrogen Bacteria with Ku-1 Cation Exchange Resin During Chemosynthesis in the Presence of $NaHC^{14}O_3$

| Fraction | Exposure 6 min | | Exposure 2 hours | |
|---|---|---|---|---|
| | disintegrations/min · 10 mg dry bacterial substance | in % of radioactivity of aqueous solution | disintegrations/min · 10 mg dry bacterial substance | in % of radioactivity of aqueous solution |
| Aqueous extract | 9100 | 100 | 62,827 | 100 |
| Fraction containing sugars, organic acids, and phosphoric esters | 2650 | 29.0 | 15,500 | 24.7 |
| Amino acid fraction (ammonia eluate of KU-1 cation exchange resin) | 4050 | 44.4 | 36,300 | 57.8 |

soluble substances. According to other data, after five minutes of photosynthesis in the green leaves of corn, the fraction containing sugars, organic acids, and phosphoric esters constituted 61%, while the amino acids were 39% (determined by difference of counts in the solution before and after treating it with KU-1 cation exchange resin). In beans the percentages were 77.0 and 23.0, respectively (Doman and Vaklinova, 1958).

By radiochromatography of the fraction containing substances which are not absorbed by KU-1 cation exchange resin, at fairly prolonged exposures, very weakly labeled substances can be found which coincide with the location of phosphoric esters, monosugars, and malic, citric, and succinic acids (Fig. 3).

As many as ten labeled compounds could be found in the eluate from KU-1 cation exchange resin. The most radioactive of these were glutamic acid, $\alpha$-alanine, and a product found in the zone where the amino acids which mova a long distance in phenol and butanol are located (phenylalanine, leucine, norleucine, etc.) (Fig. 4).

The comparison of the assimilation products of labeled carbon as they are being formed in the dynamic state was accepted as the most suitable way to carry out a comparative study of the photosynthetic and chemosynthetic pathways of $C^{14}O_2$ fixation.

It is well known that during short-term photosynthetic fixation of labeled carbon dioxide by chlorophyll-containing organisms, a considerable part of the radioactivity is found in the phosphoric esters fraction, particularly in phosphoglyceric acid (Calvin and Benson, 1949).

By analogy with higher plants and green algae, the appearance of labeled phosphoric esters first of all could have been expected in bacteria as well. However, the initial results we obtained proved to be somewhat unexpected.

As has been reported earlier (Doman and Romanova, 1959), after short-term (3-30 sec) fixation of the $C^{14}$ of dissolved labeled sodium bicarbonate and radioautography of the labeled products obtained, insignificant radioactivity was obtained in the phosphoric esters zone. The most intensive label appeared in a substance of unknown nature, located on the chromatograms at a considerable distance from the zone of the phosphoric esters of sugars and phosphoglyceric acid.

The new way in which we set up the experiment enabled us to obtain radioactive extracts which were free of admixtures of salts, thus considerably facilitating the chromatography of the labeled products of chemosynthesis. Aside from the aforementioned advantages of this method, the rate of $C^{14}$ fixation from the gas phase proved to be considerably higher than from solution. This is also of considerable significance in improving the quality of the chromatograms.

Photographs of radiochromatograms obtained by using the solvent systems water-saturated butanol—85% formic acid (9 : 1) and butanol—glacial acetic acid—water (78 : 20 : 50) are given in Fig. 5. It is easily seen from Fig. 5 that with a 5-second exposure to labeled $CO_2$, the preponderance of the radioactivity was found in the corner of the chromatogram which was the furthest removed from the starting point. Two considerably less intense spots on the radioautograph correspond to the positions of glutamic and aspartic acids (the location of the latter coincides with that of monosaccharides). In the given case, no other fairly intensely labeled substances were found after a 5-second exposure of hydrogen microbes to $C^{14}O_2$. As the duration of exposure of the bacteria to labeled carbon dioxide was increased to 30 seconds, the relative radioactivity of the spot which traveled a long distance decreased, while radioactivity in the zone

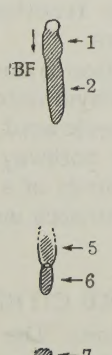

Fig. 3

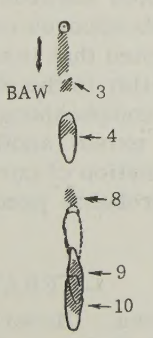

Fig. 4

Fig. 3. Radiochromatogram of neutral and acid labeled products formed after two hours of chemosynthesis by hydrogen bacteria in $NaHC^{14}O_3$. Solvent: water-saturated butanol—formic acid (95:5) (diagram). 1) Distribution zone of phosphoric esters; 2) monosugars; 3) aspartic acid; 4) glutamic acid; 5) citric acid; 6) malic acid; 7) succinic acid; 8) $\alpha$-alanine; 9) phenylalanine; 10) leucine; 11) adenine; 12) uracil; 13) first labeled product found during short-term chemosynthesis by hydrogen microbes in $C^{14}O_2$; 14) and 15) unidentified substances. Solvents: BF) water-saturated butanol—formic acid (95:5); BAW) butanol—acetic acid —water (78:20:50).

Fig. 4. Radiochromatogram of labeled products contained in eluate from KU-1 cation exchange resin after two hours of chemosynthesis by hydrogen bacteria in $NaHC^{14}O_3$. Solvent: butanol—acetic acid—water (4:1:1) (diagram). Designations same as in Fig. 3.

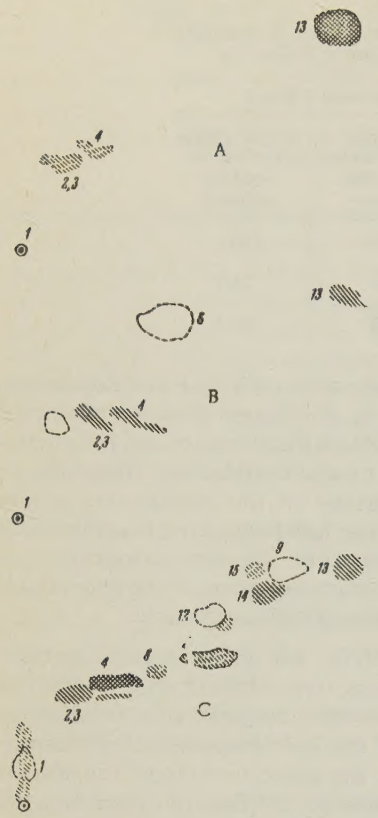


Fig. 5. Radiochromatogram of labeled products formed during chemosynthesis by hydrogen bacteria in $C^{14}O_2$. Exposure to labeled $CO_2$: A) 5 sec; B) 30 sec; C) 30 sec. Designations same as in Fig. 3.

of glutamic and aspartic acids increased. Aside from this, faint shadows of new labeled substances appeared on the x-ray film, particularly in the region of phosphoric esters. After 5 min of chemosynthesis in labeled $CO_2$, radioactivity was clearly discernable in the distribution zones of phosphoric esters and aspartic, glutamic, and citric acids, and two new radioactive spots also appeared in the far corner of the chromatogram (wih the solvent butanol—acetic acid—water) located at the level of phenylalanine.

## DISCUSSION OF RESULTS

At the time we obtained the basic results of this work, a very short communication appeared concerning an investigation in which the author (Bergman, 1957) found the main portion of the radioactivity in sugar phosphates and a small amount in malic acid when using exposures to $C^{14}O_2$ lasting 5–15 sec. On the basis of his data, the author concluded that a mechanism similar to that of photosynthesis is operative in hydrogen bacteria.

The same point of view was expressed by McFadden and Atkinson (1957), based on indirect data obtained while studying the effect of inhibitors on the autotrophic fixation of $CO_2$ by hydrogen bacteria.

Among the labeled products of chemosynthesis by Hydrogenomonas facilis, formic and acetic acids have been found recently (Orgel, Dewar, and Koffler, 1956). These last two substances could not be found by means of the methods we employed.

On the basis of the data we obtained, a conclusion concerning the complete similarity of the pathways of fixation of carbon dioxide carbon during photosynthesis and during chemosynthesis must be regarded as premature. At the present level of knowledge, three alternative hypotheses can be made concerning this question:

1. In its basic outlines, Calvin's cycle exists in the metabolism of hydrogen bacteria, but its reactions, which go on at approximately equal and rather rapid rates, do not lead to the accumulation of the characteristic products which are easily detectable during photosynthesis in plants.

2. Calvin's cycle, at least in the strain of hydrogen bacteria studied by us, plays an insignificant role in comparison with another pathway of $C^{14}O_2$ fixation connected with the early formation of substances of a basic character.

3. Finally, and least likely in the light of the data existing in the literature, is the hypothesis that Calvin's cycle as such is entirely absent in chemosynthesizing hydrogen bacteria, and that the appearance of labeled phosphorylated products is due to their secondary formation as the result of extremely intensive respiration.

## SUMMARY

1. A convenient procedure has been developed for experiments on carbon dioxide fixation (both dissolved and gaseous) by hydrogen bacteria in chemosynthesis. With this procedure, some prospects are opened for the study of the assimilation pathways of carbon in microorganisms under conditions very close to natural physiological conditions of their development.

2. One of the first intensively labeled chemosynthetic products of hydrogen bacteria is a compound located far from the zone of phosphorus esters, which are much less abundant. Chromatographic behavior and some other properties of this early product have been studied.

Among the early chemosynthetic products actively labeled glutamic and aspartic acids have likewise been revealed.

3. With rather short exposures (~5–6 min) the ratio of labeled chemosynthetic products of hydrogen bacteria is appreciably different from that of products formed upon photosynthesis in green plants at the same exposures to labeled carbon dioxide. The difference is that in the bacterial bodies radioactivity of the fraction of basic compounds is approximately equal to that of the fraction composed of neutral and acid products, while in higher plants, the ratio of these fractions is strongly shifted towards the fraction which contains sugars and phosphorus esters.

It is suggested that in addition to the carbon pathway, which is similar to the photosynthetic one via ribulose diphosphate and phosphoglyceric acid, in hydrogen bacteria there exists another pathway which is linked with the formation of compounds of a basic character, and which probably predominates under certain conditions.

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*See English translation.

# THE OXIDATION AND TRANSFORMATION OF GLUTAMIC ACID BY ACTINOMYCES VIOLACEUS

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Original article submitted December 17, 1959

While studying the amino acid composition of the culture fluid of Actinomyces violaceus, we established that this microorganism produces from four to eight amino acids during the course of fermentation on medium containing glucose and potassium nitrate (Bekhtereva, 1958). Glutamic acid was among the amino acids which we found. Free glutamic acid was also found in the mycelium.

In the present work we studied the oxidation and transformation of glutamic acid during the growth of A. violaceus in a medium where glutamic acid was the sole source of carbon and nitrogen. As is well known, glutamic acid occupies an important place in the metabolism of a number of microorganisms. The data of some authors (Bezborodov, 1958) suggest that a large amount of glutamic acid is found in the composition of the free amino acids fraction of the mycelium of various species of actinomycetes. Changes in its amount as a function of the composition of the medium were studied. It was also shown that A. griseus is capable of oxidizing glutamic acid at a rapid rate in experiments carried out in Warburg respirometers in pH 7.6 phosphate buffer (Inow, 1958).

According to our data, a medium containing glutamic acid is favorable for antibiotic biosynthesis by A. violaceus. The rate of antibiotic production is higher on this medium than on a medium with glucose. This decidedly deserves attention. Products of the transformation of glutamic acid are probably intermediate substances essential for the biosynthesis of the antibiotic molecule.

## EXPERIMENTAL PART

The oxidation and transformation of glutamic acid was studied on the mineral medium described in the preceding communication (Bekhtereva, 1958*). In this mineral medium, glucose and potassium nitrate were replaced by glutamic acid in amounts of from 0.25 to 0.5%. The media were sterilized and inoculated with A. violaceus spores. The cultures were grown in flasks on a shaker at 26-27°C. On the third and sixth days of fermentation, 25 ml of culture fluid was taken to determine organic acids and amino acids in it by the paper partition chromatography method. The acids were extracted from the culture fluid by the method described earlier (Bekhtereva, 1960).

The results of the analyses are given on chromatograms I and II.

When A. violaceus was grown on the medium indicated above for three days, along with glutamic acid (1), seven amino acids were always found: (2) alanine, (3) $\gamma$-aminobutyric acid, (4) valine, (5) phenylalanine, (6) leucine, and (7) and (8) as yet unidentified amino acids. The eighth amino acid, which was produced in large quantities from glutamate, has a high $R_f$, exceeding that of leucine and norleucine. As seen from the chromatogram, alanine and $\gamma$-aminobutyric acid were produced in very slight amounts at this stage of development. In three days, A. violaceus produced relatively large amounts of phenylalanine and leucine on medium containing glutamic acid. On the sixth day of fermentation by A. violaceus, the glutamic acid was noticeably used up. Six amino acids including glutamic acid (2) were found in the culture fluid: (1) lysine, (3) alanine, (4) leucine, and (5) and (6) unknown substances located below leucine (chromatogram II).

Judging by the size of the spots on chromatogram II, the quantitative content of amino acids had decreased relatively in the six-day culture. Valine, phenylalanine, and $\gamma$-aminobutyric acid were assimilated.

Further, the ability of the mycelium to oxidize certain amino acids in Warburg respirometers was also investigated in order to determine the assimilability of various amino acids by this microorganism. The results of the analyses are given in the table.

It is seen from the table that A. violaceus oxidized glutamic and $\gamma$-aminobutyric acids and alanine the most vigorously. Under these conditions, aspartic acid and valine were oxidized less vigorously.

In a preceding work we showed that potassium cyanide does not completely inhibit respiration in A. violaceus and that residual respiration always remains. This residual respiration must apparently be regarded as due to the catalytic action of flavoprotein oxidases (Mikhlin and Kolesnikov, 1947). It is known that some amino acids are oxidized by both flavoprotein oxidases and by dehydrogenases.

Glutamic acid dehydrogenase is widely distributed in animal (Quastel and Wheatley, 1932) and plant tissues (Kretovich and Drozdova, 1948; Sisakvan and Chamova, 1949), as well as in microorganisms (Euller et al., 1938). Glutamic acid dehydrogenase has been isolated in crystalline form (Olson and Afinsen, 1953).

*In Fig. 5 of the published work (Mikrobiologiya 27, No. 5 (1958) [p. 553 of English translation]) the first amino acid should be cystine, not tryptophan.

The Oxidation of Various Amino Acids by Actinomyces violaceus (100 mg of mycelium + phosphate buffer, pH 7.2 + M/30 amino acid)

| Amino acids | Oxygen consumption in liters after 2 hours | |
|---|---|---|
| | endogenous respiration | with substrate |
| *l*-Glutamic acid | 65.2 | 134.0 |
| γ-Aminobutyric acid | 60.0 | 110.9 |
| *dl*-Alanine | 63.0 | 107.6 |
| *dl*-Aspartic acid | 66.5 | 84.0 |
| *dl*-Valine | 64.5 | 83.0 |

We also studied the activity of glutamic acid de-ıydrogenase in mycelium which had been frozen and :reated with acetone. In the presence of glutamate and DPN+, the acetone preparation made from a three-day culture of A. violaceus decolorized methylene blue in 26 min. In the absence of this substrate decolorization of methylene blue was not observed in this period of :ime.

A. violaceus apparently contains glutamic acid de-ıydrogenase which dehydrogenates glutamic acid with he formation of α-ketoglutaric acid and ammonia. If his is actually so, then the culture fluid should con-ain α-ketoglutaric acid or its conversion product – succinic acid.

For this purpose organic acids were determined during the growth of the A. violaceus culture in a mineral medium to which 0.5% glutamate was added. The organic acids were extracted from the culture fluid by the method described earlier (Bekhtereva, 1960), and were determined by the paper chromatography method. The results of the analyses are given on chromatograms III and IV.

It is seen from chromatogram III that on the third day, A. violaceus produced six acids from glutamic acid alone: α-ketoglutaric, lactic, succinic, fumaric, and two acids which have not yet been identified. In the six-day culture the same acids remained, but (according to size of spots) their amounts decreased noticeably (chromatogram IV).

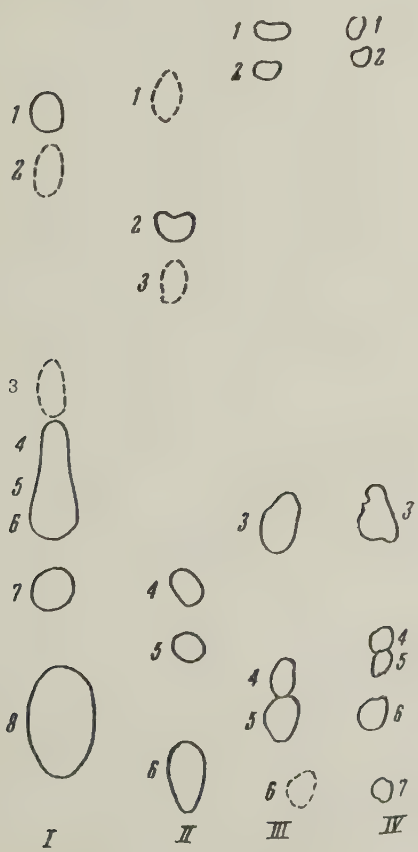
Thus these facts suggest that glutamic acid is possibly dehydrogenated to α-ketoglutaric acid by dehydrogenase. The α-ketoglutaric acid is converted to succinic acid by means of oxidative decarboxylation.

It is known that in the tricarboxylic acid cycle, α-ketoglutaric acid can leave the reaction chain by means of amination due to free ammonia (Kretovich and Bundel', 1950) and conversion to *l*-glutamic acid, or by means of the transamination reaction (Braunshtein and Kritsman, 1937).

The splitting off of amino groups from amino acids also occurs by means of transamination of α-ketoglutaric acid with the subsequent oxidation of the glutamic acid formed through the dehydrogenase and cytochrome systems.

It can be assumed that the process of glutamic acid oxidation in the A. violaceus culture apparently occurs in the following manner. In the presence of glutamic acid dehydrogenase, glutamic acid is dehydrogenated to α-ketoglutaric acid and ammonia. α-Ketoglutaric acid is oxidized through the tricarboxylic acid cycle with the production of succinic and fumaric acids. We identified all of these acids by the paper chromatography method (chromatograms III and IV). Fumaric acid can then be hydrated to malic and the latter is dehydrogenated to oxalacetic acid; oxalacetic acid is decarboxylated to pyruvic. The latter can form alanine by means of amination or by transamination with glutamic acid. Phenylalanine can be formed easily from alanine. Glutamic acid can be decarboxylated to γ-aminobutyric acid. All of these amino acids were identified by paper chromatography in three- and six-day cultures of A. violaceus during fermentation with glutamic acid alone.

Glutamic acid is not only well assimilated by A. violaceus but is a good substrate for antibiotic production. On mineral media this microorganism produces antibiotic more rapidly in the presence of glutamate than with glucose. Consequently the rate of antibiotic production in the presence of glutamic acid considerably exceeds the rate of its production on glucose.




Chromatogram I. Culture fluid after three days of fermentation by Actinomyces violaceus. 1) Glutamic acid; 2) alanine; 3) γ-aminobutyric acid; 4) valine; 5) phenylalanine; 6) leucine; 7–8) unknowns.
Chromatogram II. Culture fluid after six days of fermentation. 1) Lysine; 2) glutamic acid; 3) alanine; 4) leucine; 5–6) unknowns.
Chromatogram III. Culture fluid after three days of fermentation. 1–2) Unknown acids; 3) α-ketoglutaric; 4) lactic; 5) succinic; 6) fumaric.
Chromatogram IV. Culture fluid after six days of fermentation. 1–2) Unknown acids; 3) α-ketoglutaric; 4) unknown; 5) lactic; 6) succinic; 7) fumaric.

## SUMMARY


1. Actinomyces violaceus assimilates glutamic acid well and transforms it into a large number of compounds. Glutamic acid is the most actively oxidized of all the amino acids tested by us. This microorganism produces $\alpha$-ketoglutaric, succinic, fumaric, lactic, and three as yet unidentified acids from glutamic acid alone.

2. A. violaceus probably contains glutamic acid dehydrogenase which dehydrogenates glutamic acid to $\alpha$-ketoglutaric acid. The latter is converted to succinic acid by means of oxidative decarboxylation. Succinic acid is dehydrogenated to fumaric by succinic dehydrogenase.

3. A. violaceus produces about seven amino acids from glutamic acid alone: alanine, $\gamma$-aminobutyric acid, valine, phenylalanine, leucine, and two as yet unidentified amino acids.

4. A. violaceus produces antibiotic at a greater rate on a mineral medium containing glutamic acid than on the same mineral medium with glucose and $KNO_3$.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# THE PHOSPHORUS METABOLISM OF ASPERGILLUS NIGER VARIANT T-1 OBTAINED BY ULTRAVIOLET IRRADIATION

# III. THE METABOLIC ROLE OF PHOSPHATASE

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## INTRODUCTION

The metabolic function of phosphatases in the vital activity of animals, plants, and lower organisms has not yet been conclusively clarified. It has been established that there are not fewer than 15-20 separate enzymes belonging to this group. They catalyze a large number of important chemical reactions.

It is known that phosphatases are associated with the metabolism of carbohydrates, nucleotides, and phospholipids. In vitro experiments make it possible to pose the hypothesis that, aside from their well known hydrolytic activity, phosphatases also serve the function of transferring the phosphoric acid molecule (Axelrod, 1948; Meyerhof and Gleen, 1950).

Danielli (1951) developed this hypothesis by suggesting that soluble phosphatases, artifically extracted from organisms, behave more like phosphokinases than like phosphatases. On the one hand, phosphatases have a hydrolytic function which, in vivo, consists of the prevention of the accumulation of phosphorylated compounds by means of their hydrolysis, thus preventing the accumulation of energy in phosphate bonds (Meyerhof and Wilson, 1949). On the other hand, phophatases resemble phosphokinases in their activity, since they can transfer the reserve energy of the phosphate bond to intermediate metabolic products and prepare the latter for further enzymatic conversions.

The study of complex enzyme systems by modern methods has yielded valuable information concerning both the production of complex cell substances and the nature and fate of metabolic products. In this respect molds occupy a special position, because it is convenient to cultivate them under precisely reproducible conditions.

After Neuberg (1911) discovered active phosphatase in yeast, a large number of investigations have been carried out to study phosphatases in microbes. Investigations in this field have been conducted more recently by Sadasivan (1950, 1952). While studying the activity of phosphatase and the role of zinc in penicillin production by Penicillium chrysogenum Q-176, he found alkaline phosphatase with a pH optimum of about 8.7, the activity of which was inhibited in cyanides in a concentration of 0.002 M. The inhibition was relieved by the addition of zinc sulfate in a concentration of 0.002-0.004 M. These results give reason to suppose that the phosphatase of P. chrysogenum Q-176 is a zinc-containing enzyme.

Experiments to investigate the pyrophosphatases of molds and the polyphosphatases of P. chrysogenum Q-176 were carried out by Krishman (1951, 1952). He established the optimum pH of various phosphatases, and also studied the role of polyphosphatases in the metabolism of fungi.

Krishnan and Bajaj (1953a, b) studied the polyphosphatase system of Aspergillus niger. They found a definite connection between the activity of specific polyphosphatases and the amount of labile phosphates. Even before this investigation, however, Linderberg and Malmgren (1952), who were studying the activity of polyphosphatase during the life cycle in A. niger, came to the conclusion that no correlation exists between phosphate content and enzyme activity.

Varma and Srinivasan (1954) found that, in A. flavus mycelium, along with two phosphatases which are active at pH 2.3 and 3.6, there are two more phosphatases, one of which has an optimum for activity of pH 7.6 and the other of 8.75. These authors succeeded in establishing differences between the phosphatases by treating them with activators and inhibitors.

The present work is a continuation of our other work (Sung, 1960), where we carried out a comparative investigation of phosphorus compounds in A. niger (mutant T-1 and the original 6/5 culture). The task of the present work was to study the phosphatase of A. niger T-1, to work methods of its isolation and determination, to determine optimum conditions for enzyme activity, to study the dynamics of its activity, and to compare it with the phosphatase activity of the original strain. Aside from this we carried out a preliminary investigation of the effect adding phosphorus had on the conversion of sugar to acid and on the inhibition of phosphatase activity, as well as the connection between the latter and the distribution of the intracellular forms of phosphorus.

## EXPERIMENTAL PART

### METHODS

The method of cultivating the fungi has been set forth in detail in previously published article (Sung, 1960).

Table 1. The Effect of pH on the Phosphatase Activity of Aspergillus niger Mutant T-1 and of the Original 6/5 Strain

| pH | Orthophosphate released, $\gamma$ | |
|---|---|---|
| | T-1 | 6/5 |
| 2.1 | 235.2 | 186.7 |
| 2.7 | 201.6 | 145.8 |
| 3.2 | 152.9 | 94.4 |
| 3.7 | 102.4 | 76.8 |
| 4.7 | 64.0 | 29.6 |
| 5.6 | 16.8 | 20.0 |

Table 2. Enzyme Activity on Various Substrates

| Substrates | Concentration | Orthophosphate released, $\gamma$ | | % of control |
|---|---|---|---|---|
| | | T-1 | 6/5 control | |
| Sodium pyrophosphate | M/5 | 310 | 210 | 147.6 |
| Sodium trimetaphosphate | M/5 | 70 | 54 | 131.5 |
| Sodium glycerophosphate | M/5 | 180 | 84 | 217.0 |
| 1,6-Diphosphofructose | M/5 | 244 | 164 | 148.8 |
| Adenosine triphosphate | M/5 | 251 | 125 | 194.5 |
| Adenylic acid | M/5 | 11 | 9 | 111.5 |
| Sodium nucleate | 1% | 75 | 48 | 156.3 |

### 1. The Production of Acetone Preparations

Our experiments in which the phosphatase of the fungus was studied were carried out with acetone preparations. The solution was poured out from under the pellicle, and the pellicles were rinsed with distilled water; then anhydrous acetone cooled to 0°C was added to them with mixing. After 10-15 min the acetone was poured off. A little more cooled anhydrous acetone was added and was poured off after several minutes. The acetone-treated pellicles were dried in a desiccator over phosphoric anhydride under slight vacuum. Preparations obtained in this manner can be stored for a long time.

### 2. Extraction of Enzymes

The enzymes in the acetone preparations were extracted with water in a ratio of 1 : 25 from material previously ground to a powder. The extraction was carried out while mixing for an hour at room temperature and subsequently centrifuging.

### 3. Determination of Phosphatase Activity

The experimental mixture consisted of 2 ml of buffer and 0.5 ml of 0.01 M Na-$\beta$-glycerophosphate as the substrate (Ca-$\beta$-glycerophosphate passed through KU-2 cation exchange resin) and 0.5 ml of enzyme extract. The samples were incubated for 20 min at 37°C, after which enzyme action was stopped by the addition of 1 ml of 20% trichloracetic acid.

Control samples were set up in the same manner except that trichloracetic acid was added before the addition of enzyme. Part of the experimental mixture was then analyzed for free orthophosphoric acid content by the Fiske and SubbaRow method (Umbreit's modification, 1949). Phosphatase activity is expressed in increment of orthophosphate in $\gamma$/mg of acetone preparation in 20 min.

## EXPERIMENTAL RESULTS

### I. Peculiarities of the Phosphatase Reaction in A. niger Strain T-1, obtained as the result of UV Irradiation, and in the Original 6/5 Strain

The dependence of phosphatase activity on pH. As is well known, phosphatase has a characteristic optimum pH value depending on the substrate on which it acts. Furthermore the same enzyme, but of different origin, may have different pH optima when acting on the same substrate.

It is seen from the data presented in Table 1 that the optimum for the activity of the enzymes from both strain T-1 and strain 6/5 lies within an unusually acid zone (pH of approximately 2.1). Activity is absent in the alkaline zone. These data are in sharp distinction from those known from the literature. Proceeding from these observations it was of interest to determine whether the pH optimum for the activity of the given enzyme corresponds to the pH value during the period of cultivation of the fungus.

Alteration of enzyme activity on various substrates. As seen from Table 2, the cell-free extract of acetone-treated mycelium can split inorganic pyrophosphate, trimetaphosphate, fructose diphosphate, adenosine triphosphate, and sodium nucleate, and does not catalyze the hydrolysis of adenylic acid. This shows that the cell-free extract which we obtained from the

Table 3. The Dynamics of the Phosphatase Activity of the Fungus Aspergillus niger Strains T-1 and 6/5 During the Course of Growth (increment of orthophosphate in $\gamma$ per mg of dry acetone preparation in 20 min)

| Expt. No. | Medium | Strains | Age of fungus (days) | | | | |
|---|---|---|---|---|---|---|---|
| | | | 2 | 3 | 4 | 5 | 7 |
| 1 | I | T-1<br>6/5 | 3.98<br>5.55 | 3.00<br>1.71 | 2.40<br>1.45 | 2.50<br>1.05 | 0.50<br>0.48 |
| | II | T-1<br>6/5 | —<br>— | 4.81<br>4.87 | 5.20<br>4.94 | 6.71<br>5.82 | 7.10<br>8.26 |
| 2 | I | T-1<br>6/5 | 5.05<br>5.55 | 5.31<br>3.62 | 4.69<br>3.07 | 3.75<br>2.07 | 1.62<br>1.42 |
| | II | T-1<br>6/5 | 5.00 | 5.30<br>5.45 | 5.17<br>4.76 | 7.15<br>5.91 | 6.92<br>8.20 |
| 3 | I | T-1<br>6/5 | 11.9<br>5.9 | 5.5<br>2.3 | 4.6<br>2.1 | 4.2<br>1.3 | —<br>— |

Table 4. The Effect of the Addition of Phosphorus on Phosphatase Activity in Strain T-1 (increment of orthophosphate in γ per mg of dry acetone preparation in 20 min)

| Expt. No. | Medium | Phosphorus | Age of fungus (days) | | |
|---|---|---|---|---|---|
| | | | 2 | 4 | 6 |
| 1 | I | 0.05 % $KH_2PO_4$ | 6.80 | 5.49 | 2.21 |
| | II | (control) without phosphorus | — | 6.85 | 6.93 |
| | | 0.05% $KH_2PO_4$ | — | 5.25 | 3.62 |
| 2 | I | 0.05 % $KH_2PO_4$ | 7.80 | 4.80 | 1.80 |
| | II | (control) without phosphorus | — | 7.30 | 4.50 |
| | | 0.05 % $KH_2PO_4$ | — | 15.30 | 19.30 |

Note. The sign — designates that the determination was not performed.

acetone preparation acts differently on different substrates. The activity of the enzyme of strain T-1 was higher than from strain 6/5 on all substrates (ranging from 31.5-117.0%).

II. Dynamics of Fungal Phosphatase Activity at Various Stages of Growth

Experiments to study the dynamics of phosphatase activity were carried out with two strains, T-1 and 6/5. For this purpose the fungi were cultured by the usual method with the replacement of the nutrient solution by a solution of sugar with 0.2% $NH_4Cl$ after two days from the moment of inoculation. Analyses were performed after various intervals of time. The pellicles were treated with acetone (method indicated above).

Tests for phosphatase activity were started on the second day following inoculation when an extremely thin pellicle spread over the surface of the liquid medium. In all experiments (Table 3) phosphatase activity of the first medium (i.e., on mineral medium with sucrose) reached a maximum on the second day counting from the moment of inoculation, and then decreased as the age of the fungus increased. After the pellicle was transferred from the nutrient medium to the sugar solution, the phosphatase activity increased greatly.

Despite the difference in the ability of these strains to synthesize citric acid, the dynamics of the activity of their enzymes basically retained similar patterns under the given conditions of cultivation. However if the phosphatase activities of strain T-1 and strain 6/5 are compared, it can be seen that the activity of mutant T-1 was greater than that of the original 6/5 on both medium I and medium II. This fact agrees with our first article (Sung, 1960) concerning the fact that T-1 consumes phosphorus from the medium more intensively per unit of biomass during growth than does 6/5.

In familiarizing the reader with the first series of experiments set up for the purpose of elucidating the dynamics of phosphatase in fungi, we drew attention to the fact that on medium I phosphatase activity decreased as the age of the fungus increased, but that it increased on the sugar solution (medium II). The question necessarily arises whether this increase in activity on medium II is connected with the addition of sugar solution under the pellicle. It could be supposed that the high phosphatase activity on medium II is explained by the redistribution and regeneration of phosphorus compounds in the pellicle which had been produced during growth on the first, phosphorus-containing medium.

Our preceding results (Sung, 1960) pertaining to the study of the redistribution of phosphorus compounds in mycelium after transfer of the pellicle to medium II show that secondary redistribution of the phosphorus compounds accumulated by the mycelium during growth on medium I actually occurs.

III. The Effect of Phosphorus on the Inhibition of Phosphatase Activity, the Synthesis of Intracellular Phosphorus Compounds, and the Conversion of Sugar to Acid

Proceeding from the assumption that phosphatase plays a role in the secondary redistribution of phosphorus compounds accumulated by the mycelium during growth on medium I, we added phosphorus to the sugar solution containing 0.2% $NH_4Cl$. We assumed that the presence of phosphorus in the external medium should cause the fungus to assimilate it, as the result of which the rate of mobilization of mycelial phosphorus would decrease.

As seen from Table 4, in the case where phosphorus was added to medium II the phosphatase activity of strain T-1 dropped greatly in comparison with medium II without added phosphorus. In order to clarify the nature of this phenomenon we carried out an analysis of the different forms of phosphorus in the pellicle of strain T-1. As seen from Table 5, a considerable increase in the amount of acid-insoluble polyphosphates occurred in the pellicles during growth on sugar solution with phosphorus. An increase in the stable phosphate of the acid-soluble fraction was also observed. When the pellicle was transferred to medium II with phosphorus, intensive accumulation of fungal biomass occurred (Table 6). This correlates well with the increase in the amount of nucleic acids occurring under these conditions (see Table 5).

In our experiments, aside from determining the activity of the phosphatases, we determined total acidity, residual phosphorus, and sugar in the solution from under the pellicles (see Table 6). Four days following the transfer to medium II with phosphorus the fungus developed a large biomass but produced very little acid. A corresponding decrease in sugar

Table 5. Amounts of Various Forms of Phosphorus in the Cells of Strain T-1 After Transfer of the Pellicle to Sugar Solution with Phosphorus

| Phosphorus | 20% sucrose + 0.2% $NH_4Cl$ (control) | | | | 20% sugar + 0.2% $NH_4Cl$ + + 0.05% $KH_2PO_4$ | | | |
|---|---|---|---|---|---|---|---|---|
| | Fourth day | | Sixth day | | Fourth day | | Sixth day | |
| | γ/mg dry wt | % total P | γ/mg dry wt | % total P | γ/mg dry wt | % total P | γ/mg dry wt | % total P |
| Total | 7.02 | 100 | 5.68 | 100 | 8.36 | 100 | 6.97 | 100 |
| Acid-soluble | 2.25 | 32.0 | 2.38 | 42.0 | 2.73 | 32.7 | 3.30 | 47.4 |
| Inorganic | 0.89 | 12.6 | 0.89 | 15.6 | 0.73 | 8.7 | 0.69 | 9.9 |
| Labile | 0.75 | 10.7 | 0.55 | 9.7 | 0.68 | 8.2 | 0.69 | 9.9 |
| Stable | 0.61 | 8.7 | 0.94 | 16.5 | 1.32 | 15.8 | 1.92 | 27.6 |
| Phospholipids | 0.82 | 11.7 | 0.86 | 15.2 | 0.78 | 9.3 | 0.88 | 12.6 |
| Nucleic acids | 1.41 | 20.1 | 1.10 | 19.3 | 1.93 | 23.1 | 1.40 | 20.1 |
| Nonnucleic phosphorus (polyphosphates) | 1.90 | 27.1 | 0.45 | 7.9 | 2.75 | 32.9 | 0.87 | 12.5 |
| Residue after extraction with $HClO_4$ "phosphoproteins" | 0.54 | 7.7 | 0.89 | 15.7 | 0.95 | 11.4 | 0.52 | 7.5 |

consumption occurred. During growth on medium II without phosphorus the reverse relationship was observed – the production of a considerably smaller amount of mycelium and the accumulation of a large amount of acid with increased sugar consumption. In cultures on medium II with phosphorus a considerably larger percent of carbohydrate was conberted to acid as compared to the control without phosphorus.

## DISCUSSION OF RESULTS

Phosphatase activity was maximal in the two-day pellicle. At this time the content of phosphorus per mg of dry pellicle also reached a maximum (Sung, 1950). Then as the age of the pellicle increases, the phosphatase activity drops and the phosphorus content of the mycelium also decreases correspondingly. This means that in the vital activity of the fungus, phosphatase is closely connected with the dynamics of the phosphorus content of the mycelium. When sugar solution was poured under the pellicle, the phosphatase activity increased as the age of the fungus increased. It was shown (Sung, 1960) that when the pellicle was transferred from nutrient medium to sugar solution, the phosphorus compounds in the pellicles which had been synthesized by the fungi on medium I containing mineral phosphorus were mobilized and underwent various changes, and in this case the hydrolytic function and the function of transfer were very actively brought about by the phosphatase. As a consequence of this, phosphatase activity increased greatly following the transfer from medium I to medium II. If phosphorus is added to the sugar solution, the process of mobilization of internal phosphorus is absent. In this case phosphatase is not utilized and its activity decreases.

When phosphorus is added to the sugar solution, aside from the drop in phosphatase activity, the yield of citric acid is also reduced and the consumption of sugar also decreases correspondingly, but the synthesis of biomass becomes greater. We attempted to treat these phenomena from the point of view of deviant metabolism and assumed that the synthesis of citric acid requires particular conditions of fungal metabolism. The addition of mineral phosphorus to the medium in all probability disrupts the normal metabolism of the fungus for acid synthesis. Normal enzyme systems are suppressed, while supplementary enzyme systems which are usually in the latent state are activated. Therefore the direction of metabolism diverges from acid synthesis. The presence of an appropriate amount of phosphorus in the pellicle serves as a factor regulating the conversion of sugar to acid. When there is an excess of phosphorus absorbed from the medium in the pellicle, the conver-

Table 6. The Effect of the Addition of Phosphorus on the Conversion of Sugar to Acid and on Phosphatase Activity (data on the sixth day of growth of strain T-1 with replacement cycle of development)

| Experiment No. | Medium with 20% sugar and 0.2% $NH_4Cl$ | Acid production on medium, mg | Sugar consumption per ml of medium, mg | Conversion of sugar to acid in % of initial concentration of sugar in medium | Residual phosphorus per ml of medium in % of initial concentration of phosphorus in medium | Weight of pellicle when grown on 25 ml of medium, mg | Phosphatase activity, increment of orthophosphate in γ/mg of dry acetone preparation in 20 min |
|---|---|---|---|---|---|---|---|
| 1 | Without phosphorus | 111.0 | 160.8 | 65.4 | – | 475.6 | 19.3 |
| | 0.05% $KH_2PO_4$ | 24.6 | 78.0 | 33.4 | 65.6 | 547.5 | 4.5 |
| 2 | Without phosphorus | 88.3 | 112.0 | 73.3 | – | 359.8 | 15.8 |
| | 0.05% $KH_2PO_4$ | 35.2 | 65.0 | 49.0 | 51.8 | 448.8 | 5.6 |

sion of sugar to acid diminishes and even stops, while active synthesis of fungal biomass begins.

The phenomenon of the inhibition of phosphatase activity by the addition of phosphorus to the medium and the activation of the synthesis of internal phosphorus compounds under the influence of added phosphorus shows that after the transfer of pellicles to sugar solution with phosphorus, polyphosphates are synthesized vigorously in them. This is particularly apparent in the change in the amount of acid-insoluble polyphosphates (see Table 5). The polyphosphate content increased parallel to the increment in the amount of nucleic acids.

The author expresses deep appreciation to Prof. A. A. Imshenetskii and E. L. Ruban for valuable guidance in the work.

SUMMARY

1. The pH optimum of Aspergillus niger phosphatase lies within the acidic zone (~ 2.1) both in the initial 6/5 strain and in the T-1 mutant obtained through UV irradiation. No alkaline phosphatase has been found.

2. A study of phosphatase activity at various stages of fungal development in medium I showed that while being maximal in young films, it decreases in both strains with increase in the age of the culture.

3. In medium II, where the fungi intensively produce citric acid, the phosphatase activity in fungal films greatly increases.

4. Phosphatase activity both in the nutrient medium and in a sugar solution is always higher, at various stages of development, in the T-1 mutant than in the initial 6/5 strain.

5. The addition of phosphorus to the sugar solution causes in the T-1 mutant a decrease in the yield of citric acid and an increased synthesis of the biomass.

6. Addition of mineral phosphorus to the sugar solution results in a rapid drop of phosphatase activity in the T-1 mutant.

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# THE ISOLATION OF PURE CULTURES OF NITROSOMONAS FROM VARIOUS NATURAL SUBSTRATES AND THEIR CHARACTERISTICS

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During the last 40 years, the formation of a new branch of general microbiology—the ecology of microorganisms—has been occurring.

The ecological approach for studying the functional and morphological properties of microbes is being more widely incorporated into microbiology all the time. Great importance is being accorded to the conditions of the habitat of microorganisms in nature, and the necessity for detailed study of the metabolism of microorganisms and of the environment to which they are adapted is being emphasized. Numerous investigators have shown that microorganisms inhabit a great variety of natural substrates and are frequently encountered in places where it would seem that conditions for their growth do not exist at all (mountain peaks, ocean depths, desert sands). Nitrifying bacteria, putrefactive bacteria, butyric acid bacteria, and various species of actinomycetes and molds are especially widely distributed. Thus, for example, in soil samples from Europe, Asia, America, and Africa, different investigators (Vinogradskii, 1952; Jensen, 1951; Meiklejohn, 1949, 1954, and others) found nitrite and nitrate bacteria everywhere.

Since, in various substrates, a number of environmental factors are maintained at a certain level over a long period of time, an adaptive variability in microbes results. Distinct races appear among them which are adapted to the given conditions of existence.

Consequently, the environmental conditions of microorganisms have a decisive effect on their development.

The subject of our investigation on the ecological adaptation of microorganisms is the nitrifying bacteria which, as is well known, have a prominent role in the nitrogen cycle of nature. Extensive material on the physiological, biochemical, and morphological properties of these bacteria has now been gathered. However, from the ecological point of view, they have been studied very little (Vinogradskii, 1952; Jensen, 1950; Rubentschik, 1929; Rubel', 1913; Neelov, 1903, and others).

Our investigations were devoted to the study of the ecological properties of nitrite bacteria isolated from various habitats (various soils, manures, active sludge). For this, it was first necessary to obtain enrichment and pure cultures of Nitrosomonas from the given subsrtates and to study their morphological and physiological properties.

In our work, soil samples differing in their genesis and chemical characteristics were used; these soils were collected from various soil-climatic zones of the Soviet Union and the Bulgarian National Republic. Soil samples for the isolation of nitrite bacteria were collected in the following areas of the Soviet Union: Kamennaya Steppe, the Tellerman Experimental Forestry Station, and Southern Karelia (near the city of Petrozavodsk). In the Bulgarian National Republic, samples were collected in the neighborhood of the city of Karnobat (South Bulgaria) and in the small town of Sitnyakovo (the mountainous region of Bulgaria).

The chemical characteristics of the soil samples examined are given in Table 1.

Samples of active sludge, as well as of cow and horse manure, were taken for comparison with the soil samples.

The air tanks of the Lyublino Station for purifying sewage in the Moscow region provided the active sludge. It contained 43.39% carbon and 7.2% nitrogen (by dry weight). The cow manure was taken from a collective farm (the town of Pushkino in the Moscow region), while the horse manure was taken from a farm of the Timiryazev Academy of Agriculture (Moscow).

Of the ten soil samples, six were collected twice in different seasons and they were also analyzed twice. The samples of active sludge were also collected twice.

The isolation of enrichment and pure cultures of Nitrosomonas from various natural substrates was preceded by the determination of their content of nitrite bacteria (by the limiting dilutions method). Inoculations were made on various liquid mineral media and on media prepared from extracts of the corresponding samples of natural substrates.

The following liquid mineral media were the most frequently used in the work: 1) Vinogradskii's (1952), 2) Meiklejohn's (1950), 3) medium with double ammonium-magnesium phosphate salt, and 4) liquid mineral media to which various amounts of extracts of the substrates being analyzed were added, as well as 0.1% solution of peat humates.

The extracts were prepared by steeping 0.5 kg of each of the various substrates in 1 liter of tap water for 12 hours, and were sterilized for 20–25 minutes at 1 atm. The ratio of substrate to water was 1 : 2; 0.05% $K_2HPO_4$, 0.1% $(NH_4)_2SO_4$, and several drops of chalk

*Data are given for 1 g of soil and manure and 1 ml of active sludge

Table 1. Chemical Characteristics of the Samples of Natural Substrates Investigated

| Samples of natural substrates investigated | Depth, cm | pH | Total C, % | Humus, % | Total N, % | Water-soluble organic substances (in % of total C of soil) |
|---|---|---|---|---|---|---|
| 1. Ordinary chernozem, Kamennaya Steppe | 0–28 | 6.28 | 4.79 | 8.26 | – | – |
| 2. Lumpy-prismatic, soda-sulfate solonetz, Kamennaya Steppe | 3–20 | 7.7 | 4.73 | 8.16 (0–10 cm) 7.53 (10–18 cm) | | 1.3 (0–10 cm) 1.1 (10–18 cm) |
| 3. Dark-gray forest soil, Tellerman Experimental Forestry Station | 3–10 | 6.9 | – | 12.20 | – | – |
| 4. Basaltiform solonetz of a steppified glade in the forest (same place) | 0–5 | 7.5 | – | 3.88 | – | – |
| 5. Slightly leached chernozem in steppe under forest strip of oak (same place) | 0–10 | 7.26 | – | 9.82 | 0.45 | – |
| 6. Sandy soil in pine forest (same place) | 0–10 | 5.5 | – | 2.0 | – | – |
| 7. Weakly podzolized soil on early alluvial sand, Southern Karelia | 0–8 | 5.13 | – | 6.15 | – | – |
| 8. Leached chernozem — peat under broad-leaved oak forest Southern Bulgaria | 0–5 | 7.8 | 6.46 | 14.36 | 0.63 | – |
| 9. Dark-colored high-mountain soil under fir woods, mountainous Bulgaria | 0–10 | 5.8 | 7.69 | 20.42 | 0.21 | – |

suspension (per 100 ml of extract) were added to the extracts. Qualitative reactions for ammonia (Nessler's reagent) and nitrous acid (Tromsdorf's reagent) were used to follow the course of the process of nitrification in the nutrient media.

The results of the experiments showed that the natural substrates analyzed represent media which differ in quality with regard to the growth of Nitrosomonas in them.

The substrates which proved to be the richest in the number of nitrite bacteria were active sludge ($1 \cdot 10^{10}$), manure ($1 \cdot 10^8$—cow manure; $1 \cdot 10^6$—horse manure), various chernozems ($1 \cdot 10^4$), dark-grey forest soil ($1 \cdot 10^3$; $1 \cdot 10^4$), and soda-sulfate solonetz ($1 \cdot 10^3$; $1 \cdot 10^4$). Thus, it can be seen that favorable conditions for the growth of Nitrosomonas are created in the given substrates.

A small number of nitrite bacteria was found in basaltiform solonetz ($1 \cdot 10^2$) and in various sandy soils ($1 \cdot 10^2$). High mountain soil proved to be the poorest in nitrifying bacteria. The number of Nitrosomonas in it was considerably less than in all the rest of the samples ($1 \cdot 10^1$). Obviously, less favorable conditions for the growth of nitrite bacteria are created in these substrates, especially in high mountain soil.

It is well known that the process of nitrification in soils depends not only on the presence or absence of the microbes themselves, but also on a number of other important factors which exert considerable influence on the course of this process (genetic type of soil, vegetable cover, moisture of the soil, climatic conditions of the locality, organic content of the soil, degree of its acidity, reserves of litter, rate of decomposition of the nitrogenous reserves of the soil, deficiency of easily mobilized nitrogen, etc.). This explains the different contents of nitrite bacteria in the soils examined.

We next proceeded to obtain enrichment cultures of Nitrosomonas. We considered cultures which were greatly enriched by nitrite bacteria, but still containing contaminants, to be enrichment cultures. They were obtained by means of repeated transfers on liquid and solid mineral media. Five to eight transfers were usually made, and after this, cultures which were greatly enriched by Nitrosomonas cells (oxidizing a total of up to 0.5–1 g of ammonium sulfate) served as the initial material for obtaining pure cultures. The extent of enrichment of the cultures by Nirtosomonas cells and the gradual decrease in the number of contaminants were also judged on the basis of microscopic preparations.

A total of 101 enrichment cultures of Nitrosomonas was isolated in the course of the work from various natural substrates (Table 2).

Extensive work with enrichment cultures isolated from various natural substrates showed that the contaminants in the enrichment cultures esamined were representatives of the genera Pseudomonas (P. fluorescens, P. nitrifaciens) and Mycobacterium (M. rubrum, M. flavum).

Myxobacteria were encountered rarely and only on silica gel. Each of the enrichment cultures contained only one of the above-mentioned contaminants.

Sporogenous bacteria, Sarcina, and representatives of other systematic groups of microorganisms were never encountered as contaminants of enrichment cultures of Nitrosomonas. A number of authors (Mekhtieva, 1954; Imshenetskii, 1955; Ruban, 1955) have pointed out the homogeneity of the contaminants of Nitrosomonas with regard to their systematic position and to the fact that they belong to the nonsporogenous bacteria.

Table 2. The Number of Enrichment and Pure Cultures of Nitrosomonas Isolated from Various Natural Substrates

| Substrates from which the cultures were isolated | Number of enrichment cultures of Nitrosomonas isolated | Contaminants in enrichment cultures of Nitrosomonas | Number of pure cultures of Nitrosomonas isolated by various methods | | |
|---|---|---|---|---|---|
| | | | drop method | consecutive dilutions method | decantation of sediment method |
| Active sludge from air tanks | 10 | Pseudomonas | 17 | 4 | – |
| Cow manure | 5 | " | 5 | – | – |
| Horse manure | 5 | " | 4 | – | – |
| Chernozem under mixed grasses in forest strips | 6 | " | 12 | 2 | – |
| Chernozem – peat under oak forest | 11 | Mycobacterium flavum | 10 | – | – |
| Chernozem under a forest strip of oak | 11 | Pseudomonas nitrifaciens | 13 | 3 | – |
| Lumpy-prismatic soda-sulfate solonetz | 10 | Pseudomonas fluorescens | 12 | – | – |
| Basaltiform solonetz from a steppified glade in the forest | 9 | The same | 9 | – | – |
| Dark-gray forest soil | 11 | " " | 13 | 2 | – |
| Sandy soil in a pine forest-moor | 6 | " " | 13 | 3 | – |
| Sandy soil in pine forest | 6 | Pseudomonas | 10 | 1 | – |
| High mountain dark-colored soil under fir woods | 11 | Mycobacterium rubrum | 12 | 2 | 3 |
| Total | 101 | | 130 | 17 | 3 |

The further isolation of pure cultures of Nitrosomonas was carried out by the following methods:

1. The drop method, using a special micropipette which has a capillary opening and a polished tip (Komarova's micropipette, 1949). The principle of this method lies in the fact that when there are very small numbers of cells in the culture fluid, it is possible to place such small drops of fluid (with a diameter of 0.2–0.3 mm) on sterile fragments of cover glasses, that some of them will contain only one or two Nitrosomonas cells, and no contaminating bacteria. Nearly all of the Nitrosomonas cultures with which the work was carried out (130 cultures) were obtained by this method.

2. The consecutive dilutions method—from highly enriched Nitrosomonas cultures, by means of which 17 pure cultures were obtained.

3. The method of repeated washing of sediment remaining when the culture fluid is decanted from enrichment cultures. It was assumed that Nitrosomonas cells which had attached themselves to particles of chalk could be free of contaminating bacteria. Three pure cultures of Nitrosomonas were obtained by means of this method.

A total of 150 pure cultures of Nitrosomonas was isolated from various natural substrates during the course of the work (Table 2).

In the isolation of pure cultures from enrichments, a great deal of attention was given to checking the purity of the cultures. Only when one has truly pure cultures of nitrite bacteria is it possible to form a clear idea of the ecological specificity and physiological properties of Nitrosomonas cultures obtained from various natural habitats.

It is well known that it is very difficult to obtain a number of microorganisms in pure culture (myxobacteria, sulfur bacteria, anaerobic cellulose bacteria). This pertains particularly to pure cultures of nitrifying bacteria, which are among the most difficult to isolate.

Attention has been drawn to this situation by nearly all of the investigators working with nitrifiers: Vinogradskii (1890-1891), Gibbs (1919), Nelson (1931), Engel and Skallau (1938), Kingma Boltjes (1935), Meiklejohn (1949, 1950, 1954), Heubült (1929), Mekhtieva (1954), Imshenetskii (1955), Ruban (1955).

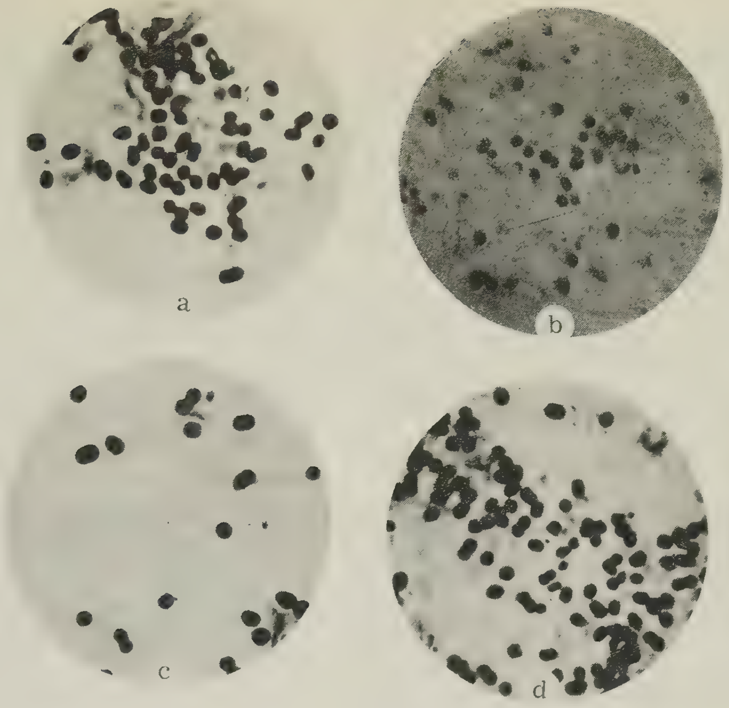
We checked the purity of the Nitrosomonas cultures by inoculating them in various nutrient media (MPB, MPB + 1% glucose, MPA, wort agar, MPG, dilute wort—7° Balling, potato agar, chopped potato, milk) to detect aerobic, facultatively anaerobic, and anaerobic bacterial contaminants of Nitrosomonas.

It is necessary to use a number of media, rather than just MPB, for checking the purity of the cultures, because certain contaminants of Nitrosomonas, such as some species of Sorangium and Mycobacterium, do not grow on meat-peptone media, as was shown in the work of Ruban (1955) and of Imshenetskii (1955). The inoculated media were kept at 24-28° for 15-20 days.

Microscopic analyses of the cultures were also performed. Preparations were made from culture fluid in which the calcium and magnesium carbonates were dissolved with dilute $CH_3COOH$ and HCl, and from the centrifuged sediment of the culture.

The preparations were stained either with Loeffler's alkaline blue or successively with basic carbol fuchsin (diluted ten times) for ten minutes and then, without washing, with 5% erythrosine (diluted ten times) for five minutes, sometimes with heating.

A study of the morphological properties of pure cultures of Nitrosomonas isolated from various natural substrates showed that they are all varieties of Nitrosomonas europaea Winogradskii. No zoogloea stage of the Nitrosomonas, i.e., large aggregates of cells of spherical form, were observed. Rod-shaped cells were also absent in pure cultures of Nitrosomonas. Pure cultures of Nitrosomonas have been similarly characterized with respect to their mor-



The morphology of pure cultures of Nitrosomonas obtained from various natural substrates (Vinogradskii's medium. Magnification 2100x). a) Nitrosomonas culture from chernozem in Kamennaya Steppe; b) Nitrosomonas culture from high-mountain soil in Bulgaria; c) Nitrosomonas culture from active sludge; d) Nitrosomonas culture from sandy soil in Tellerman.

phology by Imshenetskii and Ruban (1953), Mekhtieva (1954), and Imshenetskii (1955).

All of the pure cultures we investigated were Gram-negative, their cells showed motility, and they stained quite well with fuchsin, with erythrosine, and with Loeffler's alkaline blue. The morphology of pure cultures of Nitrosomonas showed that the cells of this species are always homogeneous.

We present a brief description of the morphology of the pure cultures of Nitrosomonas studied on liquid mineral media:

1. Culture from chernozem from Kamennaya Steppe. Cells oval, or an elongated shape, 1.5-1.7×0.7-0.9 μ.

2. Culture from chernozem from the Tellerman Experimental Forestry Station. Cells coccoid in shape, 1.6-1.8×1.2-1.3 μ.

3. Culture from Bulgarian chernozem. Cells oval, slightly elongated in shape, 1.6-1.8×0.8-1.0 μ.

4. Culture from dark-gray forest soil from the Tellerman Experimental Forestry Station. Cells oval in shape, 1.5-1.6×0.9-1.0 μ.

5. Culture from solonetz from Kamennaya Steppe. Cells oval, slightly elongated in shape, 2.0-2.2×1.2-1.3 μ.

6. Culture from solonetz of the Tellerman Experimental Forestry Station. Cells of oval, coccoidal shape, 1.9-2.0×1.3-1.4 μ.

7. Culture from sandy soil from Southern Karelia. Cells of oval, spherical form, 1.8-1.9×1.1 μ.

8. Culture from sandy soil from the Tellerman Experimental Forestry Station. Cells of oval, coccoidal form, 1.7×0.8 μ.

9. Culture from high-mountain soil from Bulgaria. Cells oval, spherical, 0.7-0.8×0.4-0.6 μ.

10. Culture from cow manure. Cells of oval, coccoidal form, 2.0×1.3 μ.

11. Culture from horse manure. Cells spherical, 1.1×1.2-1.3 μ.

12. Culture from active sludge. Cells oval, 1.8-2.0×0.9-1.1 μ.

As an illustration, we present microphotographs of several pure cultures of Nitrosomonas isolated from active sludge, sandy soil from the Tellerman Experimental Forestry Station, chernozem from Kammenaya Steppe, and high-mountain soil from Bulgaria.

Data on the nitrifying activity of enrichment and pure cultures of Nitrosomonas obtained from various habitats, as well as on the extent of their adaptation to organic substances, the active reaction of the medium, and other factors of the environment will be presented in the next communication.

## SUMMARY


1. One hundred and fifty pure cultures of Nitrosomonas, the inducer of the first phase of nitrification, were isolated from various natural substrates (various soils, manure, active sludge). The isolation of pure cultures was carried out by the following methods: 1) the drop method, 2) the method of consecutive dilutions from highly enriched culture, and 3) the method of repeated washing of sediment remaining after the decantation of culture fluid from enrichment cultures.

2. The isolated cultures showed no significant differences in morphology and were varieties of Nitro-

somonas europaea Winogradskii. Their cells were oval, spherical, or slightly elongated in shape. The dimensions of the cells varied in the range of 1.5-2.2×0.7-1.4 μ.

All of the cultures studied were Gram-negative, motile, and stained quite well with fuchsin, with erythrosine, and with Loeffler's alkaline blue. No zoogloeal stage was observed, and rod-shaped cells were also absent in the cultures.

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# EXPERIMENTAL INCREASE IN THE FREQUENCY OF NUCLEAR DIPOLARIZATION IN THE MYCELIUM OF HETEROKARYONS OF PENICILLIUM JANCHEWSKII

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Although the phenomenon of spontaneous nuclear diploidization in the mycelium of heterokaryons is frequently encountered in mold fungus, its utilization in experimental work is complicated by the extremely infrequent formation of diploids. In fungi of the genus Penicillium the frequency of spontaneous formation of diploids generally does not exceed $1 \cdot 10^{-6}$.

The method of treating heterokaryons with camphor vapors (Roper, 1952; Pontecorvo, 1953) to increase the frequency of diploid formation in the mycelium of heterokaryons of Aspergillus nidulans and A. niger produces only a slight increase in the frequency of diploidization. On the other hand, Ishitani (1956), by treating polynuclear conidia of heterokaryons of A. sojae and A. oryzae with UV rays, increased the frequency of diploidization a thousandfold.

## MATERIALS AND METHODS

For the production of heterokaryons we used mutations of biochemical deficiency produced in strain Penicillium janchewskii by the action of UV rays of 2600 A. All the mutants, apart from their biochemical deficiency, were distinguished from the initial strain by a slower rate of growth. Their colonies formed fewer spores and had white and yellow air spores in contrast to the green spores of P. janchewskii (Table 1).

As a rich (organic) medium (Sermonti, 1954a,b) we used Czapek's medium with 1% maize extract; as a minimal (synthetic) medium we used an unadulterated Czapek's medium; as a limiting medium we used a mixture of the rich and minimal media in proportions of 1:5.

For the production of heterokaryons we used the Roper-Sermonti method (Roper, 1952; Sermonti, 1954a,b). Spores of two strains of biochemical mutants which differed from each other in the color of their colonies and in their biochemical deficiency were sown together on a plate with a limiting medium in the proportion of 1 : 1 (30 conidia to a plate). The plates were then placed in a thermostat and incubated at 24° C. After 72-hr growth at the points of contact of the colonies of the two different strains, heterokaryonic filaments began to appear; after 120 hr or 144 hr of growth they were producing spores in abundance. Heterokaryon presence was clearly visible against the yellow and white grounds of the primary colonies of the biochemical mutants because of the green color of the air spores.

To obtain diploids, conidia of heterokaryons were sown on plates with the minimal medium (a million conidia per plate). The diploidization of the resultant colonies was assessed by the following: (1) their prototrophy, (2) by the increase in the size of the conidia as compared with the initial haploid strain P. janchewskii and, (3) by the diploidization of the initial strains of biochemical mutants when the spores of the diploid are sown on a rich medium.

As a factor capable of increasing the frequency of diploidization, apart from camphor vapors and UV rays, we used the action of high and low temperatures on growing colonies of heterokaryons. To test the effect of camphor vapor and UV rays heterokaryon 216 + 577 was used; for temperature treatment heterokaryons 216+577, 216+140, 216+97, 216+266, 140+577, and 577+266 were used.

When camphor was used on the lid of an upturned plate a crystal of the substance was placed on the inner side and the plates were then placed in an incubator at 36° C. for a period of 5 hr. This produced an atmosphere saturated with camphor vapor in each plate. After 5 hr the camphor was removed and further incubation of the plates was carried out at 24° C. When UV rays were used, heterokaryonic filaments (in a limiting medium on Petri plates) were irradiated directly with a dose of 2500 erg/mm$^2$. After irradiation the plates were incubated at 24° C. The cultures were usually subjected to the action of these factors after 72 hr of growth.

In order to study the effect of the action of high and low temperatures on the frequency of diploid formation the heterokaryons were exposed to a temperature of 36° C. for 1, 5, 24, 48, 96, and 144 hr; to a temperature of 42° C. for 7, 48, 72, and 96 hrs; and to a temperature of 5° C. for 48 hrs. Plates with the heterokaryonic filaments were placed in an incubator at the required temperature for the appropriate number of hours; thereafter the culture was cultivated as usual at 24° C. so that the total duration of incubation was 144 hrs.

In the course of studying the effect the age of a heterokaryon exposed to a temperature of 36° C. for 48

Table 1. Properties of Strains of Biochemical Mutants used for the Production of Heterokaryons

| Strains / Properties | Initial strain | Biochemical mutants | | | |
|---|---|---|---|---|---|
| | | 216 | 577 | 140 | 266 |
| Color of the spore-producing surface | Green | Yellow | Pale yellow | Yellow with a blue coating | White |
| Biochemical substance | – | Lysine | Arginine | Histidine | Lysine |

Table 2. Frequency of Nuclear Diploidization in Heterokaryon 216 + 577 under the Action of Camphor Vapors

| Treatment | Number of sown spores (in millions) | Number of diploids produced | Frequency of diploidization in % |
|---|---|---|---|
| Spontaneous (control) | 324.45 | 25 | $0.77 \cdot 10^{-7}$ |
| Under the action of camphor | 255.60 | 68 | $2.65 \cdot 10^{-7}$ |

Table 3. Dependence of the Frequency of Nuclear Diploidization in the Mycelium of Heterokaryon 216 + 577 on the Length of Exposure to a Temperature of 36°C

| Length of treatment (in hr) | Control | 1 | 5 | 24 | 48 | 72 | 96 | 144 |
|---|---|---|---|---|---|---|---|---|
| No. of sown spores, $10^6$ | 324.4 | 68.1 | 99.0 | 119.9 | 158.0 | 20.6 | 4.8 | 6.9 |
| No. of diploids produced | 25 | 6 | 6 | 1047 | 13300 | 1387 | 3560 | 1400 |
| Frequency of diploidization | $0.77 \cdot 10^{-7}$ | $0.87 \cdot 10^{-7}$ | $0.6 \cdot 10^{-7}$ | $0.87 \cdot 10^{-5}$ | $0.84 \cdot 10^{-4}$ | $0.67 \cdot 10^{-4}$ | $7.2 \cdot 10^{-4}$ | $2.0 \cdot 10^{-4}$ |

hr has on the frequency of diploid formation, the heterokaryonic filaments were subjected to the action of temperature at three stages of growth: (1) after 72 hr of growth when a stripe of heterokaryons is only just beginning to appear between the colonies of the biochemical mutants, (2) after 96 hr of growth when the heterokaryons are clearly visible but do not produce spores (the filaments are white), and (3) after 120 hr of growth when spores are produced in abundance (the filaments are green).

In order to verify the dependence of the frequency of reverse mutations (mutations in relation to prototrophy) on temperature, the initial strains of biochemical mutants were also subjected to the action of a temperature of 36° C. for 48 hr. Apart from this the sensitivity of the spores of different strains to the above-mentioned temperatures was also determined. For this purpose a suspension from the spores of each strain (up to 300–400 spores in 1 $mm^2$) was prepared and kept for 24 hr at 24° C. Thereafter each suspension was divided into two halves; one half was then kept for 48 hr at 36° C. and the other (the control) at 24° C. After this both suspensions were sown on plates containing the rich medium.

The treatment of heterokaryon 216+577 with camphor vapor produced only a very slight increase in the frequency of diploidization. As will be seen from Table 2 the frequency of the spontaneous formation of diploids in this combination equaled $0.77 \cdot 10^{-7}$ whereas the frequency of diploidization under the effect of camphor was only 3.4 times greater than occurred spontaneously.

The experiment has demonstrated that the method of treating heterokaryons with UV rays cannot be applied to those of Penicillium since the treatment of a heterokaryon mycelium produces a large number of reverse mutations ($1 \cdot 10^{-2}$) which renders the isolation of diploidal forms utterly impossible.

In the course of the study of the effect of high temperature on the frequency of diploidization, heterokaryon 216+577 in the first stage of growth (72 hr) was subjected to the action of a temperature of 36° C. for 1, 5, 24, 48, 96, and 144 hr.

As will be seen from Table 3, when the heterokaryons were exposed for a period of 1–5 hr to a temperature of 36° C. the frequency of diploid formation did not differ greatly from that of the control. On the other hand, when they were exposed to a temperature of 36°C. for 24, 48, 96, and 144 hr, the frequency of diploid formation increased a thousandfold compared with the control. The differences in the frequency of diploidization dependent on the duration of temperature action were no longer apparent after 24 hr of exposure.

The nature of the colonies produced was considered to be diploid on the following grounds: (1) Prototrophic

Table 4. Dimensions of spores of the Different Strains

| Strain | Diameter of spores in $\mu$ | Volume of spores in $\mu^3$ |
|---|---|---|
| P. janchewskii | 3.41 | 20.5 |
| 216 | 3.39 | 20.5 |
| 577 | 3.05 | 14.6 |
| 216/577 | 4.02 | 33.6 |

Table 5. Frequency of Diploidization of the Initial Biochemical Mutants in the Diploids 216/577

| Strain | 216/577 | 216 | 577 |
|---|---|---|---|
| Number of colonies | 16020 | 12 | 5 |
| Frequency of diploidization, % | — | $7{,}5 \cdot 10^{-2}$ | $3{,}1 \cdot 10^{-2}$ |

Table 6. Results of the Action of a Temperature of 36°C for a Period of 48 Hr on the Biochemical Mutants

| Strain | Number of sown conidia | Number of prototrophs produced |
|---|---|---|
| 216 | $8 \cdot 10^4$ | 0 |
| 577 | $1.8 \cdot 10^6$ | 0 |

Table 7. Dependence of the Frequency of Nuclear Diploidization in the Mycelium of the Heterokaryon 216 + 577 on Temperature (Exposure for a period of 48 hr)

| Temperature | 5° | 24° | 36° | 42° |
|---|---|---|---|---|
| Number of sown spores, $10^6$ | 27.5 | 324.4 | 158.7 | 2.0 |
| Number of diploids produced | 0 | 25 | 13300 | 0 |
| Frequency of diploidization, % | 0 | $0.77 \cdot 10^{-7}$ | $0.84 \cdot 10^{-4}$ | 0 |

Table 8. Dependence of the Frequency of Diploidization on the Age of the Heterokaryon 216 + 577

| Stage of growth | 3 days | 4 days | 5 days |
|---|---|---|---|
| Number of sown diploids, $10^6$ | 48.0 | 93.0 | 68.0 |
| Number of diploids produced | 783 | 270 | 0 |
| Frequency of diploidization, % | $1.63 \cdot 10^{-4}$ | $0.3 \cdot 10^{-4}$ | 0 |

Table 9. Frequency of Diploidization in the Mycelium of Different Heterokaryons. (Temperature of 36°C for a Period of 48 Hr)

| Strains | 216+577 | 216+140 | 216+97 | 216+266 | 140+577 | 577+266 |
|---|---|---|---|---|---|---|
| Frequency of spontaneous diploidization | $0.77 \cdot 10^{-7}$ | $0.33 \cdot 10^{-7}$ | 0 | $0.77 \cdot 10^{-7}$ | 0 | 0 |
| Frequency of diploidization on exposure to temperature | $0.84 \cdot 10^{-4}$ | $0.8 \cdot 10^{-5}$ | $0.75 \cdot 10^{-6}$ | $2.41 \cdot 10^{-6}$ | $0.15 \cdot 10^{-4}$ | $3.0 \cdot 10^{-4}$ |
| Increase in frequency of diploidization | 1000 times | 200 times | Over 1000 times | 30 times | Over 1000 times | Over 1000 times |

Table 10. Sensitivity of Different Strains to Temperature Treatment (at 36°C for 48 Hr)

| Strain | Number of sown spores | Survival as a percentage of those that survived at 24°C |
|---|---|---|
| 577 | 102 | 63.0 |
| 216 | 362 | 37.7 |
| 216/577 | 1505 | 84.0 |
| P. janchewskii | 2209 | 96.0 |

colonies produced by sowing spores of heterokaryons were morphologically similar to the strain P. janchewskii; the diploid differed from the initial strain only in that the color of its colonies underwent a change: The colonies of the diploid had a pale yellow coating whereas colonies of P. janchewskii were blue-green in color; (2) Spores of prototrophic diploids were considerably larger than spores of the initial strain of P. janchewskii and spores of biochemical mutants (Table 4). However, the most significant indication of the diploid nature of the prototrophic colonies produced by sowing spores of a heterokaryon subjected to temperature treatment on a rich medium is the diploidization as the result of sowing of the initial strains of biochemical mutants. The frequency of diploidization is shown in Table 5.

In the control tests exposure of the initial strains 216 and 577 to a temperature of 36° C. for a period of 48 hr did not result in a single prototrophic colony (Table 6).

Exposure to a temperature of 42° C. and 5° C. produced no increase in the frequency of diploidization (Table 7).

Tests carried out to verify the dependence of the frequency of diploid formation on the stage of growth of a heterokaryon exposed for 48 hr to a temperature of 36° C. have shown that the action on the heterokaryon at the stage when spores are present in abundance is not effective (Table 8). On the other hand, temperature treatment of a heterokaryon during the first and second stages of growth (white filament) produces in both instances a thousandfold increase in the frequency of diploidization.

Table 9 shows that under the action of high temperature a 30- to 3000-fold increase occurred in the frequency of diploidization of all the heterokaryons verified.

## DISCUSSION

The increase in the frequency of diploidization which Roper obtained by the use of camphor vapors and which our experiments have confirmed is explained by him in terms of the lysis of the hyphae of the mycelium under the action of camphor. Haploid hyphae undergo lysis more rapidly than diploid, which gives the latter a certain selective advantage. As a result the number of diploid nuclei in the mycelium increases.

The presence of lysis in the mycelium under the action of a high temperature gave us reason to suppose that a high temperature without the use of camphor could be used as a selective agent.

As the results demonstrate, exposure to a temperature of 36° C. for a prolonged period (24 to 144 hr) produces a substantial increase in the frequency of diploidization. The fact that the exposure of a heterokaryon to a temperature of 36° C. for 1–5 hr has no effect can probably be explained by the insufficient time for the lysis of the mycelium. A temperature of 42° C. has the same disastrous effect on both the haploid and diploid hyphae. Naturally in these conditions

the latter do not possess a selective advantage. This also explains the negative result produced by exposure to low temperature (5° C.) insofar as no lysis of the mycelium occurs under these conditions.

In order to verify the assumption regarding the selective action of high temperature, we determined the sensitivity to temperature of four strains: P. janchewskii, diploid 216/577, and biochemical mutants 216 and 577. All four strains were exposed to a temperature of 36° C. for a period of 48 hr. Under these conditions the diploid really did have a certain selective advantage over the biochemical mutants (Table 10). However, this advantage (only two times greater in the case of strain 216 and still less in the case of strain 577) cannot explain the thousandfold increase in the frequency of diploidization. That is why, without discarding the hypothesis of the selective advantage of diploid nuclei in the mycelium of the heterokaryon when the latter is exposed to high temperature, we also have to assume high temperature exerts an effect directly on the mechanism of nuclear diploidization in the mycelium of the heterokaryon.

## SUMMARY

1. Treatment of the heterokaryon with camphor vapors produces a slight increase in the frequency of diploid formation.

2. The method of UV-ray treatment of heterokaryons is not suitable for Penicillium heterokaryons.

3. Exposure of heterokaryons to high temperature at the stage of spore formation produces an increase in the frequency of diploidization which varies with different combinations from 30 to 3000 times.

4. Such an increase in the frequency of diploid formation produced by high temperature is probably due to the formation in the mycelium of the heterokaryon of a selective advantage of the diploid nuclei insofar as the temperature sensitivity of biochemically deficient nuclei is greater than that of the diploid nuclei. However, the temperature can probably influence the diploidization process in a direct way as well.

5. This procedure can be used for the experimental production of diploids in Penicillium.

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# VOLUTIN IN ACTINOMYCETES AND ITS CHEMICAL NATURE

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A large number of granules is formed in the mycelium of Actinomyces aureofaciens and A. rimosus when the latter are cultivated on media rich in phosphorus. The granules can be stained metachromatically with methyl and toluidine blue and resemble volutin granules of fungi and microorganisms (Prokof'eva-Bel'govskaya and Popova, 1959; Zaitseva and Mikhailova, 1959). In the mycelium of A. streptomycini the formation of volutin can be provoked by the action of an actinophage (Peshkov, Rautenshtein, Sorokina, Cherednichenko, Sharkova, 1952).

In the majority of industrial strains of A. streptomycini and A. aureofaciens the appearance of volutin is accompanied by a sharp fall in the capacity of the mycelium to biosynthesize an antibiotic (Bel'govskaya and Zolotnitskii, 1951; Peshkov, Rautenshtein, Sorokina and Sharkova, 1951, and others) and that is why the study of the conditions of volutin formation is of great practical interest.

The chemical nature of volutin in actinomycetes has been insufficiently studied. Guberniev, Torbochkina, Listvinova, and Kats (1959) have demonstrated that in the mycelium of A. aureofaciens there is a direct dependence between the number and size of the volutin granules and the amount of polyphosphates which are not soluble in cold chloric or trichloroacetic acids.

The purpose of this investigation was to study the conditions of volutin formation in a submerged culture of A. aureofaciens, its relation to the formation of an antibiotic, its morphology, the basic properties and chemical nature of the volutin granules and the changes which they undergo in the course of the development of the culture. These investigations, as we have already demonstrated, will help to clarify the physiological role of volutin in actinomycetes and will indicate the ways of preventing its mass formation in industrial fermentations, thereby increasing the antibiotic productivity of the mycelium.

## MATERIALS AND METHODS

For the purpose of this investigation we used four industrial strains of A. aureofaciens: LS-536, LS-112, LS-40, and LS-B-16 produced by the selecting laboratory of the All-Union Scientific-Research Institute of Antibiotics. The retorts were inoculated with spores which had been maintained on millet. The spores were sown on a synthetic medium of the following composition: starch, 2.5%; $(NH_4)_2SO_4$, 0.55%; $KH_2PO_4$, 0.02%; $MgSO_4$, 0.05%; $ZnSO_4$, 0.005%; $CuSO_4$, 0.005%; NaCl, 0.2%; $CaCO_3$, 0.4%; pH 6.8, and were cultivated for a period of 30 hr at 28° C. in Erlenmeyer retorts with a capacity of 750 ml and containing 125 ml of medium. The retorts were placed on a circular rocking device with a rotating speed of 200 rpm. On a synthetic medium of the above composition but containing different amounts of orthophosphate (from 0.01 to 0.2%), starch (from 1.2 to 7.5%) and ammonium sulfate (from 0.2 to 1.0%), three percent of mycelium was sown and cultivated for a period of 96 hr.

Fixation of the mycelium was carried out in several ways: in vapors of a 2% osmic acid (2 min), with a Carnoy medium (10 min), with 10% formalin pH 6.8-7.0 (10 min). For staining volutin we used the special methods recommended for volutin granules of other microorganisms: staining with Loeffler blue (an alcohol solution of methylene blue followed by treatment with weak acid and alkali) and by Albert's method (1921) (an alcohol solution of toluidine blue and methylene green with acetic acid) and other methods. RNA in volutin was determined by the Einarson (1951) method using a 1% solution of pyronine on a phosphate buffer pH 4.2 and gallocyanin-chromic alum both before and after treatment with ribonuclease; to determine DNA Feulgen's reaction and treatment with deoxyribonuclease was used. To detect polyphosphates we used the methods of Wachstein and Pisano (1950) and those of Ebel, Colas, and Muller (1958); both methods with $Pb(NO_3)_2$ followed by treatment with $(NH_4)_2S$ and Lindegren's method (1951) (a water solution of toluidine blue with lactic acid). For staining nuclear elements we used the Robinow method as modified by Peshkov in 1955 (i.e., Giemsa solution after hydrolysis in HCl and subsequent staining with light green). Apart from this, nucleic acids and polyphosphates were extracted separately by chloric acid by the method of Erickson et al. (1948) or by trichloroacetic acid by the Schneider method. Microphotographs were taken by a microphotographic apparatus FMN-3 (homal IV, obj. 90×, virtual image apochromatic) with orange and green filters.

## RESULTS

### a) Morphology of Volutin Granules and their Interrelation with Nuclear Elements

Mass formation of volutin in strain LS-112 was observed after 48 hr of growth on a synthetic medium with 0.08% orthophosphate. The volutin granules in such a mycelium were clearly visible in vivo in a

phase-contrasting microscope. Against the background of a light cytoplasm they appeared as dark sharply outlined structures of a rounded slightly elongated biscuit shape unequally distributed along the length of the hyphae. Often the volutin granules were situated practically at the very tip of the growing end of the hyphae. In vital staining with a water solution of neutral red (1:5000) pH 7–7.2 they did not change color.

When the mycelium was fixed in a Carnoy medium and stained with methylene or toluidine blue the volutin granules had distinct outlines and stood out as red-violet, generally quite metachromatic structures against a pale blue or pale violet cytoplasmic background.

The shape of the volutin granules varied both within the limits of the same microcolony as well as within the same hyphae. Together with punctuated rounded granules, oval, biscuit-shaped, highly diffuse as though gemmulated or severely elongated granules were also not infrequently encountered (Fig. 1a, 2a). Sometimes the protoplasm of the hyphae was so heavily overladen with volutin that individual granules became merged, forming long strap-shaped structures (Fig. 3b,c).

The sizes of individual granules of a microcolony varied within the range ($0.2-2.2\times0.2-0.8\mu$). The diameter of the volutin granules at times considerably exceeded the thickness of the hyphae (Fig. 1a).

The volutin granules had sharp edges and appeared to be surrounded by a film. This was observed both in live unstained as well as on the fixed stained mycelium. On grinding the quickly frozen mycelium with sand or aluminum oxide the volutin granules did not lose their structure. This shows that they possess great strength.

In the mycelium of strains LS-536, LS-112, and LS-B-16, which had been stained with methylene blue, nuclear elements of a blue color could sometimes be discerned together with red-violet volutin granules. In most cases, however, they could not be seen at the same time. To clarify the interrelation between these cellular elements microphotography was done on an identical section of the culture of strain LS-112 after 72 hr of growth on a synthetic medium with 0.06% $KH_2PO_4$. The mycelium was then stained with methylene blue (Fig. 3e; the nucleoids were almost invisible, the volutin was stained metachromatically) and by the Robinow method (Fig. 3f; volutin granules dissolved as a result of hydrolysis in HCl at 60° C. but the nucleoids were clearly visible). On comparing the two photographs it will be seen that the volutin granules were situated at considerable distances from each other and as a rule between elongated or rounded groups of nucleoids. In some cases the volutin gran-

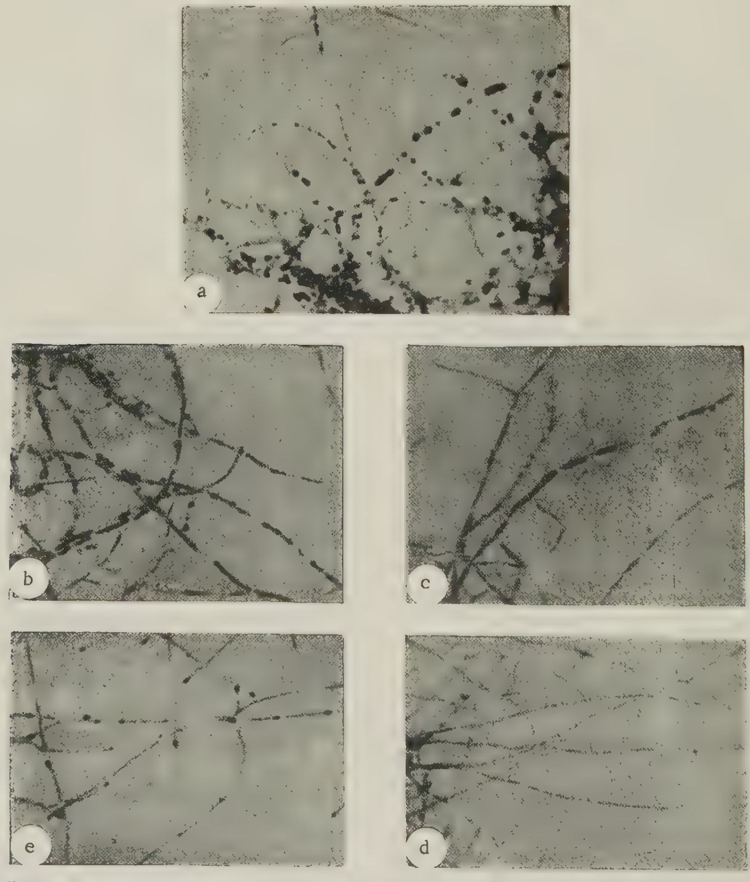


Fig. 1. *A. aureofaciens*. Strains LS-112 and LS-40. (a) Synthetic medium with 0.08% $KH_2PO_4$, 48 and (b) 72 hours of growth. Fixing agent: Carnoy. 2000x. a,b) Methylene blue; c) the same after treatment with hot water (for control see b); d,e) staining by the Meyer method; after treatment with 1% $H_2SO_4$ (d), after treatment with 4% $K_2CO_3$ (e).

ules appeared to be incorporated in a group of small nucleoids.

This shows that the volutin granules and nucleoids do not produce actinomycetes of a single morphological complex in the hyphae as claimed by Bringmann for corynebacterium and actinomycetes (1950–1951) but exist in the hyphae simultaneously and independently of each other although not infrequently the distances between them are small.

(b) Basic Properties and Chemical Composition of Volutin

To study the chemical composition of volutin we used the mycelium of strain LS-112 or strain LS-40 which had been cultivated for 48 hr on a synthetic medium with 0.08% $KH_2PO_4$. Such a mycelium contained many large volutin granules which when stained with methylene blue were highly metachromatic (Fig. 1a). The metachromasis did not disappear after the action of weak acids. This property of volutin manifested itself when the mycelium was stained by the Meyer method (Fig. 1d,e). The volutin dissolved rapidly in hot water and weak alkali (Fig. 1c,e) but not in alcohol and chloroform.

Volutin granules were thoroughly stained by all the principal dyes: neutral red (only after fixation), toluidine blue, pyronine, gentian violet, and others. The high basophilia of the volutin granules depends to some extent at least on the presence therein of nucleic acids. On treating the mycelium with ribonuclease certain granules dissolved completely while others decreased in size but retained their capacity for metachromasis (Fig. 2a,b). When the mycelium was treated with 10% cold chloric acid for a period of 18 hr (to remove RNA) the same results were obtained as in treatment with ribonuclease. Other evidence of the presence of RNA in volutin granules of A. aureofaciens was the positive staining with pyronine and gallocyanin-chromic alum. Pyronine stained the volutin granules purple; in cases where the mycelium had been pretreated with ribonuclease the staining was negative. When the pH was 0.8–0.9 gallocyanin-chromic alum stained the highly metachromatic volutin a reddish color and the nucleoids blue. When the pH was 1.2–1.4 the metachromasis of volutin became more intense. It did not disappear when the mycelium was pretreated with ribonuclease but the number of granules in the hyphae as well as the size of the granules decreased (Fig. 2c,d). This shows that the volutin granules contain different amounts of RNA.

The fact that the volutin granules were partially dissolved by ribonuclease and cold chloric acid and were stained by pyronine and gallocyanine-chromic alum when the pH was 0.8–0.9 shows that RNA was present in the volutin of A. aureofaciens.

Apart from RNA the volutin granules also contained other compounds comprising the residue after RNA had been extracted with cold chloric acid or eliminated by fermentation with ribonuclease. The capacity of the residual bodies to be stained metachromatically indicated the presence therein of polyphosphates. However, the metachromatic effect can be produced both by polyphosphates and by acid mucopolysaccharides, the difference being that the metachromasis of the polysaccharides disappears when treated with an acid whereas that of the polyphosphates remains (Ebel, Colas, and Müller, 1958). That is why in order to determine the presence of polyphosphates in volutin granules we used metachromatic staining of volutin with toluidine blue in an acid medium by the Lindegren method. Most volutin granules were thoroughly stained by this method (Fig. 2e). When treated with cold trichloroacetic acid the granules were stained as in the control; when treated with hot trichloroacetic acid the staining disappeared (Fig. 2f). Two other methods of detecting polyphosphates, those of Wachstein and Pisano (1950) and Ebel et al., are based on the fact that the polyphosphates form insoluble complexes with lead salts. By these methods the volutin granules of A. aureofaciens were stained black and stood out clearly against the bright pink cytoplasm which had been stained with safranine. After preliminary treatment with cold chloric acid (which removed the RNA and acid-soluble polyphosphates) the staining was positive; after treatment with hot chloric acid (which removed the DNA and the acid-insoluble polyphosphates) the staining was negative.

Thus in addition to RNA the second component of volutin granules of actinomyces is acid-insoluble polyphosphates.

To determine the presence of DNA in the composition of volutin of A. aureofaciens we stained the mycelium with volutin by the Feulgen method but when subjected to hydrolysis the volutin granules dissolved completely. When the mycelium was treated with desoxyribonuclease and stained with methylene blue the size and appearance of the granules did not change but remained the same as in the control (for control we used mycelium stained with methylene blue but not treated with an enzyme).

To determine the presence of fat in the volutin of the mycelium fixed with formalin we stained it with sudan III. In this instance volutin granules were not stained but when the mycelium containing volutin was fixed in osmic acid vapors darkening of volutin, indicative of the presence of fats, was not observed.

Thus the volutin granules of A. aureofaciens contain neither DNA nor fats; they are formed by the RNA complex and by acid-insoluble polyphosphates.

(c) Heterogeneity of Volutin Granules

When the culture of strain LS-B-16 was cultivated on a synthetic medium with an excess of inorganic phosphorus (0.2% of orthophosphate) a large number of volutin granules appeared in the mycelium. Observation of the granules during the process of development of the culture showed that their shape, size, and chemical composition changed according to the age of the hyphae. After 18–24 hr of growth, volutin granules were either totally absent or else present as small rounded granules widely spaced (Fig. 3a). They were stained blue by methylene blue without metachromasis and were almost completely dissolved by ribonuclease. These properties of volutin granules of young mycelia show that the granules are formed principally by RNA.

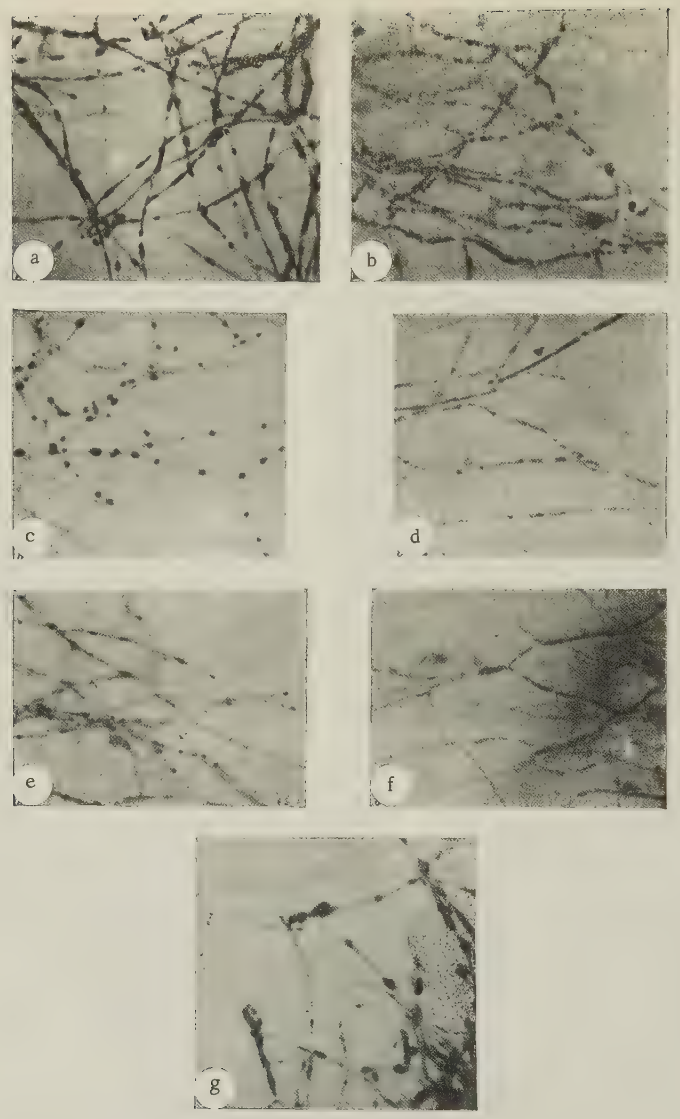

Fig. 2. A. aureofaciens. Strain LS-112. Synthetic medium with 0.08% $KH_2PO_4$ (a-f) and a medium with sunflowerseed oil (g). 48 hr growth. Fixing agent: Carnoy. 2000x. a) Methylene blue; b) the same after treatment with ribonuclease; c) gallocyanin-chromic alum pH 0.8-0.9; d) the same after treatment with ribonuclease; e,g) stained by Lindegren method; f) the same after treatment with a 5% trichloroacetic acid at 90°C for 15 min (for control see e).

After 48 hr of growth the number of volutin granules in the protoplasm considerably increased; moreover, they were larger and generally not of a rounded but rather an elongated shape. They stained a red-violet color and were highly metachromatic (Fig. 3b,c). When the mycelium was treated with ribonuclease many volutin granules left substantial residual bodies which were formed by the polyphosphates (this was demonstrated by their metachromasis in an acid medium and by their being completely dissolved by hot trichloroacetic acid). When the culture underwent autolysis (after 96 hr of growth) the total amount of volutin was severely reduced but a considerable proportion of the granules became large. In old hyphae they stained a pink or raspberry color but the metachromasis was weaker than after 48 hr growth. Because autolysis of the volutin granules occurred later than that of the cytoplasm of the hyphae the volutin granules appeared to lie outside the hyphae (Fig. 3d). The volutin granules of autolyzed hyphae were partially destroyed by ribonuclease, which shows that they consisted chiefly of polyphosphates.

In mycelium with large amounts of volutin the differences between the granules in the central older parts of the microcolonies and those in the peripheral ends of young hyphae were always clearly visible. In the peripheral hyphae the volutin granules were usually smaller, more sparsely distributed, and stained blue-violet by methylene blue; moreover, their metachromasis was weaker than that of granules in hyphae of the central part of the microcolonies. In these latter hyphae the granules were larger and the metachromasis stronger; moreover, they stained a pink or raspberry color by methylene blue and were only partially

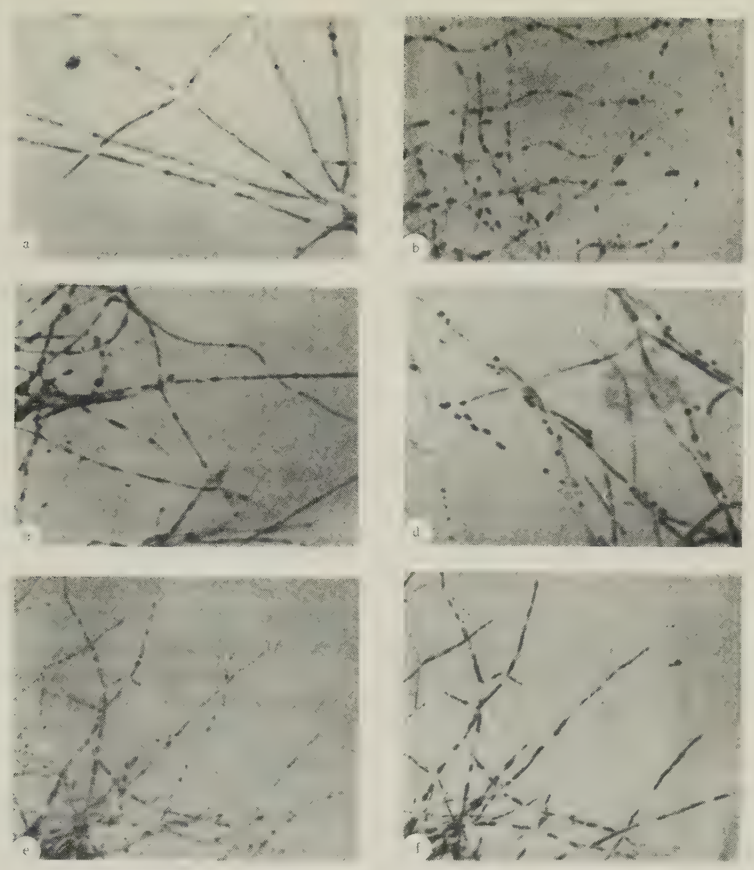

Fig. 3. A. aureofaciens. Strain LS-B-16 (a-d) and LS-112 (e,f). Synthetic medium with $KH_2PO_4$. Fixing agent: Carnoy. 2000x. a,b,c,d) 0.2% $KH_2PO_4$; (a) after 24 hr growth; (b) after 48 hr growth; (c) after 72 hr growth; (d) after 96 hr growth; (e,f) 0.06% $KH_2PO_4$ after 72 hr growth; (e) methylene blue; (f) the same site after staining by the Robinow method.

destroyed by ribonuclease; consequently they contained much greater amounts of polyphosphates than granules of peripheral hyphae.

The above shows that the chemical composition of volutin in hyphae of different ages was not the same. In the early stages of development the volutin granules contained more RNA than polyphosphates; in later stages of development polyphosphates formed the principal component of volutin.

Nevertheless it is possible to speak of the heterogeneity of volutin granules in another sense as well. Small volutin granules were stained to the same degree by methylene and toluidine blue and gallocyanin. Large granules when stained in the same way gave rise to "vacuoles" which either did not stain at all or turned a pink or raspberry color (the remaining portion of the granule stained a blue-violet color). When the Lindegren method, which is specific for polyphosphates, was used, only the "vacuoles" of the granules were stained (Fig. 2g). The color disappeared on treating the mycelium with hot chloric acid. All this shows that in large volutin granules the polyphosphates are not infrequently located in the central part of the granule and RNA concentrated around them. In small volutin granules these substances are distributed uniformly.

(d) Conditions of Formation of Volutin in A. aureofaciens

We investigated the effect of phosphorus, carbon, and nitrogen nutrition on the formation of volutin in the mycelium of A. aureofaciens on a synthetic medium.

When the concentration of inorganic phosphorus in the synthetic medium was increased ($KH_2PO_4$ from 0.01 to 0.2%) the amount of volutin in the mycelium of A. aureofaciens increased and at times reached an enormous mass. However, the sensitivity of different strains to the same concentrations of phosphorus was different. In strain LS-536 volutin granules appeared when the medium contained 0.030-0.035% $KH_2PO_4$; in strain LS-40 at 0.04%; in LS-112 at 0.04-0.064%; in LS-B-16 at 0.14-0.20% of $KH_2PO_4$. With the exception of this last strain where no decrease in activity occurred, the mass formation of volutin was accompanied by a sharp reduction in the synthesis of the antibiotic (Guberniev, Torbochkina, Kats, 1959).

In the next series of tests we investigated the effect different amounts of nitrogen (ammonium sulfate or casein) and starch in the synthetic medium had on the formation of volutin in strain LS-536. The amount of ammonium sulfate and casein in the medium ranged from 0.2-1.0%, starch from 1.2-7.5%. It was found that when the amount of organic or inorganic nitrogen was

reduced in relation to starch or when the content of starch exceeded that of ammonium sulfate the growth of the mycelium and the mass formation of volutin granules were greatly inhibited.

We next investigated the effect on volutin formation of a culture medium as it becomes more acidic. For this purpose the strain LS-536 was cultivated on a synthetic medium with chalk (control) and without chalk. In the test variant after 30 hr of growth the culture medium reached a pH of 6.0–6.2 (in the control after 36 hr, pH 6.6). As the medium increased in acidity growth was severely inhibited and the mass formation of volutin granules began; the RNA content in the cytoplasm of the mycelium was low from the beginning of development; the ability of the culture to form chlortetracycline was also severely reduced (to 112 units/ml; in the control 879 units/ml).

## DISCUSSION

The study of the basic properties of volutin of A. aureofaciens has demonstrated its similarity to volutin of fungi, algae, protozoa, and bacteria. Volutin granules of actinomycetes dissolve completely in hot water, weak alkali, 1 N HCl at 60° C., but, like volutin granules of other microorganisms, do not dissolve in alcohol and chloroform. They are highly basophilic and are metachromatically stained by methylene blue; moreover, the metachromasis does not disappear after the action of weak acids. These properties are also characteristic of volutin granules of microorganisms, fungi, and algae (Guilliermond, 1903; Meyer, 1904; Meisel', 1950; Drews, 1955; and others). Volutin of actinomycetes as well as volutin of other microorganisms is readily stained by the special methods recommended for this purpose (Loeffler blue, and by the Meier, Ernest, Lindegren, Vakhshtein, and Pisano methods).

Volutin of A. aureofaciens can be observed in vivo. In this respect it is similar to volutin of most microorganisms (Drews, 1955; Duguid, Smith, and Wilkinson, 1954) and unlike that of fungi, where it has the appearance of a vacuolar colloid which falls out in the form of granules only when it is properly stained or fixed (Guilliermond, 1903; Zikes, 1922; Meisel', 1950).

Volutin granules of A. aureofaciens form an independent structural element and are similar neither to the nuclear apparatus, as suggested by Knaysi, Hillier, and Fabricant (1950), nor to mitochondria (Mudd, Winterscheid, Delamater, and Henderson, 1951). This does not exclude the possibility of a profound functional connection between the nuclear elements and the volutin granules as suggested by Bringmann (1950–1951).

A cytochemical analysis has shown that volutin of A. aureofaciens contains both RNA and polyphosphates. The ribonucleic nature of the granules is demonstrated by the positive staining with pyronine and gallocyanin when the pH is 0.8–0.9 and by the fact that they are partially dissolved by cold chloric acid and ribonuclease. The findings regarding the high content of RNA in the volutin of actinomycetes agrees well with the data obtained for other microorganisms; for bacteria (Belozerskii, 1945; Minck and Minck, 1949; Drews, 1955), for corynebacteria (Bringmann, 1950–1951), for yeast (Meisel' and Korchagin, 1952).

Polyphosphates are also a component of the volutin granules of A. aureofaciens, which is of great interest, inasmuch as they are accumulators of energy quite as powerful as ATP (Meyerhof, Shatas, and Kaplan, 1953; and others). Cytochemical indications of the presence of polyphosphates are the metachromatic staining of volutin by methylene blue without its disappearing under the action of weak acids as well as the lead nitrate reaction in an acid medium. The results obtained by treating the mycelium with hot and cold chloric acid with subsequent staining of the polyphosphates leads to the conclusion that volutin is formed only by acid-insoluble polyphosphates. According to Belozerskii and Kulaev (1957) acid-insoluble polyphosphates play an important role in the synthesis of protein and nucleic acids.

Evidence that polyphosphates are one of the principal components of volutin in other microorganisms has been furnished by a number of other investigators (Lindegren, 1951; Smith, Wilkinson, and Duguid, 1954; and others).

The results of chemical analysis have likewise demonstrated that two of the components of volutin of A. aureofaciens are RNA and acid-insoluble polyphosphates (Guberniev, Torbochkina, and Kats, 1959).

DNA and lipids were not found in the composition of volutin granules of actinomycetes. In bacteria and corynebacteria these substances are apparently present in volutin (Drews, 1955; Ferrata, Rondanelli, Zangaglia, 1956; Bringmann, 1950–1951).

Volutin in A. auroefaciens, as in other microorganisms, is not an indispensable structural element of the cell. Volutin granules generally appear at later stages of development of the culture under conditions of disturbed balance between individual components of the nutrient medium. Our investigation of the volutin granules of actinomycetes confirms the numerous findings regarding the existence of a direct correlation between the content of inorganic phosphorus in the medium and the number and size of volutin granules (Smith, Wilkinson, and Duguid, 1954; Prokof'eva-Bel'gorskaya and Popova, 1959, and others). Nevertheless the mass appearance of volutin cannot be considered as a reaction specific only for inorganic phosphorus in the medium. Intensive formation of volutin in A. aureofaciens was also observed in media with an unfavorable condition of acidity, i.e., when there was an excess of carbohydrates in relation to nitrogenous substances or when there were insufficient nitrogenous substances in relation to carbohydrates. The formation of volutin in A. aureofaciens is thus promoted not by change in the absolute amounts of individual components of the nutrient medium but by the general lack of balance, which destroys the nucleic acid metabolism and consequently the normal development of actinomycetes. The formation of volutin in the mycelium is a reverse process, i.e., the principal components of volutin can be used in the process of development. In this instance there is no diminution of the synthesis of chlortetracycline.

## SUMMARY

In Actinomyces aureofaciens volutin granules can be observed in vivo. They exist in the hyphae independently of the nuclear elements without, as a rule, forming with them a single morphological complex. The principal components of volutin are RNA and acid-insoluble polyphosphates. The correlation between them varies with age (in young mycelium RNA predominates in the volutin; in the old, polyphosphates). Neither DNA nor lipids were found in volutin. Generally volutin granules are formed in the mycelium at advanced stages of development when the medium contains an excess of inorganic phosphorus or of carbohydrates or when there is an insufficiency of nitrogen compounds in relation to carbohydrates. In most industrial strains of A. aureofaciens mass volutin formation is associated with a decrease in protein synthesis which varies from one strain to another. Volutin formation is a reverse process, i.e., its principal components can be consumed by the mycelium in the course of growth. In such cases no decrease in activity occurs.

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# THE EFFECT OF MALONATE ON THE RESPIRATION AND OXIDATIVE PHOSPHORYLATION OF YEAST

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Investigations carried out at various times with the aid of different inhibitors have shown that the partial inhibition of respiration in yeast leads to an increase in their content of energy-rich phosphates.

In certain cases the inhibitors used acted not only on the enzymes of the terminal stage of respiration but also on other enzymes participating in anoxy- and oxybiotic transformations of sugar. Among such inhibitors is sodium fluoride, which, besides inhibiting the action of some hematin enzymes, according to existing concepts affects the action of enolase and adenosine triphosphates as well (Malkov and Bogdanova, 1956). Evidence indicates some respiratory inhibitors are also protoplasmic poisons. Among these are 2,4-dinitrophenol (Malkov and Suprunenko, 1958), sodium azide (Malkov and Leoninok, 1959), and others.

In the present investigation the effect of malonic acid on oxidative phosphorylation in yeast and its content of energy-rich phosphates was studied.

As is well known, malonic acid is considered to be an inhibitor of succinic dehydrogenase. From this it follows that malonic acid should not inhibit the process of alcoholic fermentation. At the same time, by retarding the process of the conversion of succinic acid to fumaric, malonate should, according to generally accepted concepts, cut off the Krebs cycle and block the process of respiration.

In addition, the oxidation of succinic acid to fumaric is connected with the transfer of two atoms of hydrogen and, therefore, malonic acid should to some extent block the process of oxidative phosphorylation, i.e., the formation of energy-rich phosphates.

## METHODS

The experiments were carried out on a synthetic nutrient medium of the following composition (%): glucose—5; $MgSO_4$—0.2; $(NH_4)_2SO_4$—0.5; $KH_2PO_4$—0.4. Some experiments were set up without glucose by using a 4% solution of neutralized succinic acid as the sole carbon source. Various amounts of malonic acid were added to the media. In the experiments the second generation of ordinary pressed baker's yeast was used, or yeast which had been subjected to preliminary self-fermentation at 28-30° C. by suspending in tap water and aerating for 28-48 hr or more. This operation was carried out according to Wieland's directions (Wieland and Wille, 1935) for the purpose of removing the reserve assimilable carbohydrates contained in yeast, thus obtaining so-called "impoverished" yeast. All experiments with both ordinary and "impoverished" yeast were carried out on shakers, having access to air, at 30° C for 2 hr. After the experiment the yeast was separated out and the filtrate (medium) was analyzed for alcohol content by specific gravity of the distillate. The amount of yeast was figured by the weight method while in some experiments the yeast was counted in a Thom chamber before and after the experiment.

Table 1. The Effect of Malonic Acid on the Fermentation and Respiration of Yeast (data for 100 ml)

| Concentration of malonic acid in M | Alcohol produced in g | Increment of yeast in mg per dry material | $P_7$ found, in mg % of dry wt. of yeast | $O_2$ consumed, in mm$^3$ per 10 mg of dry yeast in 10 min | Increase in $P_7$ yield with respect to control, in % | Increase in alcohol yield in % with respect to control | Inhibition of respiration of yeast in % with respect to control |
|---|---|---|---|---|---|---|---|
| Experiment 1 | | | | | | | |
| Control | 1.99 | 216.5 | 215.0 | – | 0 | 0 | – |
| 0.0001 | 1.99 | 208.0 | – | – | – | 0 | – |
| 0.001 | 1.99 | 202.5 | 224.0 | – | 4.18 | 0 | – |
| 0.01 | 2.05 | 178.0 | 262.0 | – | 21.86 | 3.01 | – |
| Experiment 2 | | | | | | | |
| Control | 1.94 | 215.1 | 172.0 | 150.4 | 0 | 0 | – |
| 0.01 | 1.99 | 174.5 | 181.0 | 106.7 | 5.23 | 2.57 | 29.08 |
| 0.1 | 1.50 | 264.0 | 174.0 | 97.2 | 1.16 | – | 35.38 |
| Experiment 3 | | | | | | | |
| Control | 1.99 | 205.5 | 191.0 | – | 0 | 0 | – |
| 0.01 | 2.05 | 223.5 | 280.0 | – | 46.5 | 3.01 | – |
| 0.1 | 1.56 | 249.0 | 321.0 | – | 68.0 | 0 | – |

Note. The sign — shows that the determination was not carried out.

Table 2. The Effect of Malonic Acid on the Respiration of "Impoverished" Yeast

| Concentration of malonic acid in M | $P_7$ found, in mg per dry material of yeast | $O_2$ consumed, in mm per 10 mg of dry material of yeast | Inhibition of yeast respiration, in % with respect to control |
|---|---|---|---|
| Control | 251.4 | 81.3 | – |
| 0.0001 | 257.6 | 53.7 | 33.9 |
| 0.001 | 253.3 | 59.3 | 27.0 |

The phosphorus content of the nonprotein filtrate before and after hydrolysis was determined in the yeast according to Lohmann. Phosphorus was determined colorimetrically according to Briggs' method as modified by Agabal'yants and Dubrovskaya (1951). The intensity of exogenous respiration of the yeast was determined by oxygen consumption in Warburg respirometers.

## EXPERIMENTAL DATA

In the first experiments of the present investigation (Table 1), the effect of malonic acid on the fermentation and respiration of yeast was studied.

The data of the first experiment show that malonic acid in a concentration of from 0.0001 to 0.01 M did not block the fermentation process, while at a concentration of 0.01 M it even activated this process somewhat. Inhibition of alcoholic fermentation was noted only when the content of malonic acid in the medium was 0.1 M.

As the concentration of malonic acid was increased the respiration of the yeast was inhibited by 29.08% at 0.01 M; at a concentration of 0.1 M malonic acid inhibited the respiration of the yeast by 35.38%.

The data presented also show that malonic acid in a concentration of 0.1 M promotes the multiplication of yeast somewhat (data of experiments 2 and 3). At higher dilutions malonic acid did not exhibit a positive effect on yeast multiplication.

Judging from the data of the three experiments presented, the yeast which had grown in the presence of various amounts of malonic acid usually contained an increased amount of $P_7$ as compared with the control.

It can thus be concluded that malonic acid does not have an inhibitory effect on fermentative enzymes, at least in low concentrations. In the presence of increased concentrations of malonic acid the respiration of yeast is inhibited but is not cut off. Seemingly, malonic acid is an incomplete inhibitor of succinic dehydrogenase.

Later (experiment 4) 4% succinic acid previously neutralized to pH 6.0 with alkali was used as the sole carbon source. Aside from succinic acid the nutrient medium consisted of 0.5% $(NH_4)_2SO_4$; 0.4% $KH_2PO_4$; and 0.2% $MgSO_4$. Before being added to the medium the yeast was suspended in tap water and aerated for 45 hr at 30° C after which the "impoverished" yeast was settled out on a Buchner funnel to the concentration of pressed yeast.

The first experiment with succinic acid (Table 2) showed that malonate best inhibits the respiration of "impoverished" yeast at a concentration of 0.0001 M. As the concentration was increased the inhibitory effect of malonic acid decreased somewhat.

The relationship which became apparent in the experiment was further confirmed in the following experiment, in which the experimental yeast, after preliminary aeration for 26 hours, contained 267.7 mg % of $P_7$ in dry material.

The data obtained (Table 3) confirmed the results of preceding experiments on the $P_7$ content of yeast as a function of the concentration of malonic acid in the media in which the yeast was cultured. The data regarding the inhibitory effect of malonic acid on yeast respiration were also confirmed. We feel the data of the last experiment on the oxidative phosphorylation of "impoverished" yeast on succinate in the presence of malonic acid were interesting. They show that the partial inhibition of the respiration of "impoverished" yeast, i.e., yeast from which the reserve intracellular carbohydrates were removed as the result of self-fermentation under aerobic conditions, as a rule leads to an increase in the content of energy-rich phosphates in the cells and to a corresponding increase in the $P/O_2$ ratio.

We explain the pattern noted in the experiments presented, wherein the cultivation of physiologically active and "impoverished" yeast on glucose or succinate in the presence of malonic acid leads to increased energy-rich phosphates in the yeast after the experiment, by the intensification of oxidative phosphorylation by yeast with partially blocked respiration.

This phenomenon can also be explained by the inhibition of the action of adenosine triphosphatase of the yeast. The following experiment was set up in order to confirm this hypothesis.

We took 1 ml of ATP solution (containing 20 mg of ATP in 50 ml of water) + 0.5 ml of enzyme preparation (yeast juice) + 0.5 ml of acetate buffer + 2 ml of dis-

Table 3. The Effect of Malonic Acid on the Respiration and Oxidative Phosphorylation of "Impoverished" Yeast

| Concentration of malonic acid in M | Multiplication coefficient of yeast | $P_7$ found, in mg % of dry material of yeast | Increment of P in γ per 10 mg of dry material of yeast | $O_2$ consumption in $mm^3$ in 60 min per 10 mg of dry material of yeast | Inhibition of yeast respiration, in % with respect to control | $P/O_2$ |
|---|---|---|---|---|---|---|
| Control | 1.0241 | 275.8 | 8.1 | 75.4 | 0.00 | 0.0075 |
| 0.0001 | 1.0067 | 293.6 | 25.9 | 60.3 | 20.00 | 0.030 |
| 0.001 | 1.0112 | — | — | 35.4 | 53.00 | — |
| 0.01 | 0.9780 | 282.1 | 14.4 | 30.0 | 60.20 | 0.034 |

Table 4. The Effect of Malonic Acid on Adenosine Triphosphatase Activity

| Concentration of malonic acid in M | pH | ATP-ase activity in γ of P per 1 ml of medium | Increase in ATP-ase activity in % with respect to control | Inhibition of ATP-ase activity with respect to control, in % |
|---|---|---|---|---|
| Control | 3.3 | 180 | — | — |
| » | 4.4 | 165 | — | — |
| » | 5.3 | 124 | — | — |
| » | 6.2 | 108 | — | — |
| 0.01 | 3.3 | 165 | — | 9.99 |
| 0.01 | 4.4 | 125 | — | 24.24 |
| 0.01 | 5.3 | 128 | 3.22 | — |
| 0.01 | 6.2 | 114 | 5.55 | — |
| 0.001 | 3.3 | 178 | — | 1.11 |
| 0.001 | 4.4 | 163 | — | 1.21 |
| | 5.3 | 127 | 2.41 | — |

tilled water. In other test tubes, 2 ml of malonic acid solution in appropriate concentrations was added instead of water. Incubation went on for 1 hr at 35° C. Inorganic P was then determined by means of a photoelectric colorimeter.

The data presented (Table 4) show that malonic acid at pH 3.3–4.4 inhibited the ATP-ase activity of yeast. At pH 5.3–6.2 at which the yeast was cultured (experiments 1–5), on the contrary, malonic acid in a concentration of 0.01–0.001 increased ATP-ase activity. From the data presented it must be concluded that the observed increase in the energy-rich phosphate content of the yeast can not be explained by the inhibition of ATP-ase activity. The facts presented are evidence that the increased content of energy-rich phosphates in the yeast and oxidative phosphorylation are in reverse relationship to the intensity of yeast respiration. Only under conditions of partial inhibition of respiration in intact yeast was an increase observed in the content of energy-rich phosphates; this was equivalent to the intensification of the process of phosphorylation associated with partially blocked, but not intensive, respiration. The latter agrees completely with our early observations (Malkov, 1951) and is confirmed by recent data, also from work with yeast (Utter, Keech, and Nossal, 1958) according to which oxidative phosphorylation on succinate is not obligately connected with respiration.

## SUMMARY

1. Malonic acid at a concentration of 0.001–0.0001 M does not inhibit aerobic alcoholic fermentation on glucose; at a concentration of 0.01 M malonic acid stimulates alcoholic fermentation somewhat, while at a concentration of 0.1 M, it inhibits it noticeably.

2. Solutions 0.0001–0.1 M in malonic acid inhibit yeast respiration on glucose by 27.0–35.38%.

3. Malonic acid in a concentration of 0.0001–0.01 M somewhat inhibits the process of yeast multiplication under conditions of aerobic fermentation on glucose.

4. The respiration of "impoverished" yeast cultured on succinate is poorer than on glucose.

5. In medium containing succinate as the sole carbon source, malonic acid in a concentration of 0.0001–0.01 M inhibits the respiration of "impoverished" yeast by 20–60%. Depending on the physiological state of the yeast, the effect of malonic acid is lessened and in some cases yeast respiration is even somewhat activated in the presence of malonic acid.

6. The partial inhibition of yeast respiration with the aid of malonic acid promotes the increase of the P content of the yeast during its cultivation and intensification of oxidative phosphorylation ($P/O_2$) irrespective of the carbon-containing compound in the nutrient medium (glucose, succinate).

7. Malonic acid in a concentration of 0.001–0.01 M in acetate buffer promotes intensification of ATP-ase activity of the enzyme preparation (juice) from yeast at pH 5.0–6.2; at pH 3.3–4.4 the same concentrations of malonic acid inhibit the ATP-ase activity of the yeast enzyme preparation.

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# THE ACTIVITY OF SOME OXIDATIVE ENZYMES IN AN ASPERGILLUS NIDULANS VARIANT OBTAINED BY MEANS OF ULTRAVIOLET IRRADIATION

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As is well known, the nutrient substances utilized by the cell serve as energy sources. These substances are broken down as the result of a number of oxidation-reduction processes.

It was therefore of definite interest to study the behavior of the enzymes participating in the vitally important cell processes of an Aspergillus nidulans variant obtained by means of irradiation. In the present work, the activity of certain oxidative enzymes (catalase, peroxidase, polyphenol oxidase, and ascorbic acid oxidase) was studied in the original A. nidulans fungus and in the variant n/7 obtained from it by means of irradiation.

In order to compare the behavior of oxidative enzymes in different species of Aspergillus a culture of A. oryzae was also used.

It is known that with the aid of irradiation considerably altered forms of fungi can be obtained, in which the activity of oxidative enzymes as well as of enzymes carrying out synthesis is usually reduced. The result of this is the retarded growth of the fungus.

Brief data on this question were reported in the literature by Pomoshchnikova (1956) and by Loginova (1960).

It could be assumed that the activity of the enzyme catalase is especially high in Aspergillus fungi because, as is well known, this enzyme is found in all aerobically respiring cells.

The basic role of this enzyme is protective. Catalase rids the organism of the hydrogen peroxide produced during respiration. During this reaction, water and inactive molecular oxygen are given off (Kretovich, 1952; Gize, 1959). However, when peroxidase reacts with hydrogen peroxide, active oxygen is given off which can oxidize various chemical compounds. This enzyme also catalyzes various polyphenols and aromatic amines.

The action of polyphenol oxidase and ascorbic acid oxidase is very specific. The first catalyzes the oxidation of polyphenols, while the second, the oxidation of ascorbic acid.

The role of these enzymes is particularly significant in plants, as is also the case with peroxidase (Mikhlin, 1956).

## EXPERIMENTAL METHODS

The fungal cultures were first grown by the surface method on wheat decoction made of bran.

The method of setting up the experiments has been described earlier (Loginova, 1960).

The activity of the oxidative enzymes was determined by the following method: the fungal pellicle, removed from the culture fluid, was rinsed on the reverse side with sterile tap water and was dried off with filter paper, after which a known weighed amount was taken. In order to determine activity, two or three pellicles were taken which were divided into two equal halves. Enzyme activity was determined in one and dry weight in the other.

Table 1. Catalase Activity

| Culture | Age of culture (days) | pH of medium at end of expt. | Am't of $O_2$ evolved, ml | | Difference between am't of $O_2$ evolved in expt and in control, in ml | Dilution of weighed portion, in ml | Wt. of absolutely dry pellicle, in g | Am't of $O_2$ evolved, recalculated per 1 g of absolutely dry wt. of fungus |
|---|---|---|---|---|---|---|---|---|
| | | | exptl | control | | | | |
| A. oryzae | 5 | 5.93 | 8.37 | 2.79 | 5.58 | 50 | 0.4239 | 329.0 |
| " nidulans | 5 | 6.52 | 8.66 | 2.84 | 5.82 | 50 | 0.2139 | 680.2 |
| Variant n/7 | 5 | 6.10 | 8.69 | 2.51 | 6.18 | 50 | 0.2991 | 516.5 |
| A. oryzae | 6 | 6.39 | 8.89 | 3.14 | 5.75 | 50 | 0.3855 | 372.9 |
| " nidulans | 6 | 6.76 | 8.94 | 2.92 | 6.02 | 50 | 0.3589 | 419.3 |
| Variant n/7 | 6 | 6.61 | 8.85 | 2.97 | 5.88 | 50 | 0.3169 | 463.8 |
| A. oryzae | 7 | 6.83 | 9.50 | 3.36 | 6.14 | 50 | 0.5281 | 291.1 |
| " nidulans | 7 | 6.92 | 8.95 | 3.41 | 5.54 | 50 | 0.3950 | 350.6 |
| Variant n/7 | 7 | 6.99 | 8.92 | 3.46 | 5.52 | 25 | 0.2321 | 254.2 |
| A. oryzae | 8 | 6.93 | 8.15 | 2.88 | 5.27 | 50 | 0.5179 | 253.4 |
| " nidulans | 8 | 6.95 | 8.85 | 2.53 | 6.32 | 50 | 0.3905 | 404.6 |
| Variant n/7 | 8 | 6.90 | 8.49 | 2.62 | 5.87 | 25 | 0.2365 | 310.23 |

Table 2. Ascorbic Acid Oxidase, Polyphenol Oxidase, and Peroxidase Activities in Various Aspergillus Cultures

| Culture | Age of culture (days) | pH of medium | Dry wt. of mycelium, g | Ascorbic acid oxidized, mg in 30 min per 1 g of absolutely dry wt. of fungus | | |
|---|---|---|---|---|---|---|
| | | | | ascorbic acid oxidase | polyphenol oxidase | peroxidase |
| A. oryzae | 4 | 6.05 | 0.2365 | 7.67 | 8.0 | None |
| | 5 | 6.35 | 0.4053 | 6.32 | 3.73 | » |
| | 6 | 6.14 | 0.5640 | 5.40 | 4.10 | » |
| | 7 | 6.53 | 0.6939 | 4.24 | 4.07 | » |
| | 8 | 6.89 | 0.6509 | 2.96 | 2.34 | » |
| A. nidulans (original) | 5 | 6.44 | 0.4232 | 0.26 | 0.86 | 2.58 |
| | 6 | 6.69 | 0.5117 | 0.43 | 0.36 | 2.62 |
| | 7 | 6.75 | 0.5903 | 0.45 | 0.45 | 2.53 |
| | 8 | 6.97 | 0.6450 | 0.45 | 0.45 | 2.16 |
| A. nidulans | 5 | 6.46 | 0.3503 | 0.32 | 0.40 | 1.36 |
| The same | 6 | 6.67 | 0.4542 | 0.28 | 0.52 | 1.81 |
| Variant n/7 | 7 | 6.81 | 0.5598 | 0.25 | 0.21 | 1.17 |
| The same | 8 | 7.04 | 0.6496 | 0.13 | 0.60 | 0.77 |

Catalase activity was determined by the gasometric method (Ermakov et al., 1952); activity was expressed in milliliters of oxygen evolved per 1 g of absolutely dry mycelium.

A weighed portion of mycelium was ground in an agate mortar for 30 minutes in a known volume of phosphate buffer at pH 6.8 (50 or 100 ml).

For the determination, 5 ml of the suspension was taken, to which 5 ml of water and 3 ml of 0.6% $H_2O_2$ were added; the reaction lasted 3 min.

Boiled mycelium which showed no catalase activity served as the control in the experiment. A blank experiment with the same reagents, but in the absence of mycelium, was also set up.

The determination of ascorbic acid oxidase, polyphenol oxidase, and peroxidase activity was carried out by a method developed by Povolotskaya and Sedenko (1955). With the aid of this method it is possible to determine simultaneously the activities of the three enzymes indicated above. The activity of the enzymes was expressed in the same units (mg of oxidized ascorbic acid).

## EXPERIMENTAL DATA

The data on the determination of catalase in the fungal mycelium show that the highest activity of this enzyme was found in the original race of A. nidulans. The catalase of variant n/7 was less active.

The activity of the given enzyme was considerably lower in A. oryzae (Table 1).

Thus on the fifth day of growth of the fungus, catalase activity in the original form of A. nidulans as determined by the amount of oxygen evolved was equal to 680.2 ml, while in variant n/7 the activity of this enzyme was reduced by 24% (516.5 ml of oxygen evolved). A. oryzae showed the lowest catalase activity (329 ml).

There are indications in the literature that catalase is quite stable to the action of UV rays.

In Escherichia coli strain K-12, for example, after treatment with rather high dosages of UV, the activity of this enzyme decreased by only 15% (Latarjet and Coldas, 1952).

Of considerable interest are the investigations of Monod, Torriani, and Jolit (1949), who established that the addition of catalase to a suspension of irradiated E. coli K-12 cells restores their previous ability to reproduce.

These investigators propose that hydrogen peroxide accumulates in the cells as the result of irradiation and that it decomposes when catalase is added.

Our data show that, in absolute figures, catalase activity in the forms of Aspergillus studied increased gradually with the age of the fungus, but when recalculated per unit weight of the fungus this activity was considerably lower than in the first days of growth (fourth-fifth day).

In order to check whether the given enzyme partially passes from the fungal film to the culture fluid appropriate experiments were set up.

The data obtained showed that no catalase activity was found in the medium. The results of determinations of ascorbic acid oxidase, polyphenol oxidase, and peroxidase activities showed that the activities of these enzymes were somewhat higher in the original form than in the n/7 variant.

In A. oryzae the activity of these enzymes was higher with the exception of peroxidase. It should be pointed out however that the activity of these enzymes was quite low in the three cultures studied.

Data on the enzyme activities are given below; they are expressed in mg of ascorbic acid oxidized in 30 min per 1 g of absolutely dry weight of fungus (Table 2).

## SUMMARY

A comparative study of the enzyme activities of the original Aspergillus nidulans strain and of variant n/7 which was obtained from it by means of UV irradiation showed that in the latter the activity of oxidative enzymes was reduced.

High catalase activity was found in all of the fungal cultures studied (A. nidulans, A. oryzae, and variant n/7). However, in variant n/7 the activity of this enzyme was 24 lower than in the original form. The catalase activity of A. oryzae was even lower.

The cultures under investigation showed very weak eroxidase, polyphenol oxidase, and ascorbic acid xidase activities. A tendency toward reduced activity f the given enzymes could be noted in variant n/7 in his case as well.

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# EFFECTS OF DIFFERENT NITROGEN FORMS ON THE UTILIZATION OF BUTYRIC ACID BY SPECIALIZED CULTURES OF AMMONIFICATORS

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In the previous report (Bychkovskaya, 1960) it was demonstrated that three aerobic non-spore microorganisms isolated from different soil samples manifested a selective capacity to utilize butyric acid salts when other assimilable sources of carbon were present in the medium.

The isolated bacteria which from their complex of symptoms appeared to be ammonificators actively assimilated butyric acid both in a synthetic medium with mineral nitrogen as well as in the usual meat-peptone broth. At the same time it should be pointed out that in a synthetic medium with peptone as the only source of nitrogen, the assimilation of butyric acid by the above-mentioned organisms was severely inhibited.

This conflicted with the active utilization of butyrates in a meat-peptone broth and has therefore made imperative the elucidation of the effects of different forms of nitrogen on this process.

Tests were carried out in a synthetic medium of the following composition: sodium butyrate – 0.2%; $K_2HPO_4$ – 0.05%; $MgSO_4$ – 0.02%; $CaCl_2$ – 0.01%; and traces of NaCl, $FeSO_4$ and $MnSO_4$; pH was 7.0–7.2. As control we used a variant, with 0.1% $NH_4NO_3$, since this form of nitrogen had been used in previous experiments. The nitrogen source was sterilized separately and introduced into the medium before sowing in amounts so calculated that the total nitrogen content in all the variants was the same. The content of butyric acid was determined by the Duclo method.

The results are presented in Figs. 1, 2, and 3, in which the curves are grouped according to the test series.

Figure 1 shows that when $(NH_4)_2SO_4$ was present, particularly large amounts of butyric acid were used by strains Nos. 1 and 21 and in smaller amounts by strain No. 3. This can probably be explained by the greater sensitivity of the latter to the physiological after-effect of acid salts. When $KNO_3$ was present the assimilation of butyrate took place more slowly in all three strains, particularly in No. 21. These findings show that the high energy of the process in the control variant with $NH_4NO_5$ was determined in the first instance by the ammonium and not by the nitrate radical.

When alanine was present (Fig. 1) butyric acid was assimilated more slowly than in the control, particularly by strain No. 21. After 192-216 hr the medium reacted positively with ammonium; in strains Nos. 1 and 3 this reaction was slight, in No. 21 intense.

When $KNO_3$ or alanine were present, the assimilation of butyrate was delayed and the retardation was particularly pronounced in the early stages of development. The rate of utilization of butyric acid by all the variants after 192-216 hr of growth is expressed in close values: 70–84% in strain No. 1, 63–68% in strain No. 3, and 43–69% in strain No. 21.

In tests with asparagine and urea the variant with 0.05% $(NH_4)_2SO_4$ (Fig. 2) served as control. When asparagine was present butyric acid was actively broken up by all the strains; moreover, after 192-240 hr the medium reacted positively with ammonia. When urea is present the butyrate is consumed particularly fast by strains Nos. 1 and 21. The fact that the medium reacts strongly to ammonium shows that the strains possess active urease.

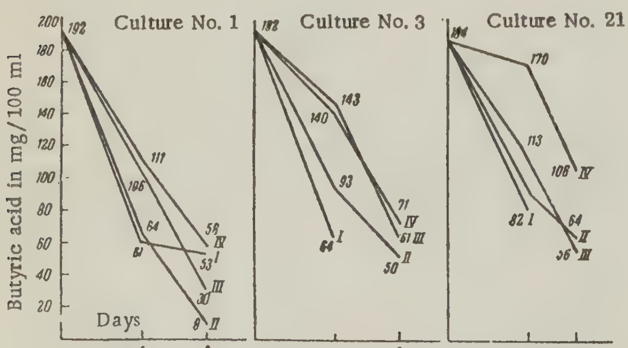


Fig. 1. Assimilation of butyric acid in the presence of I) $NH_4NO_3$; II) $(NH_4)_2SO_4$; III) Alanine; 4) $KNO_3$.

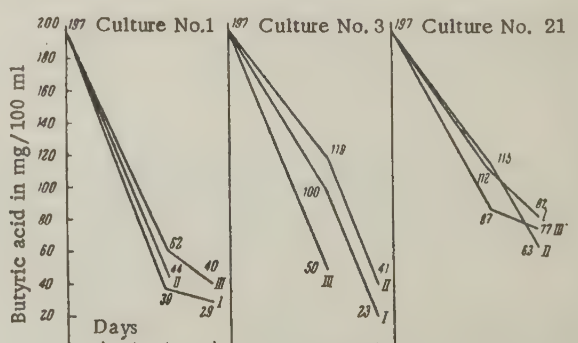


Fig. 2. Assimilation of butyric acid in the presence of I) $(NH_4)_2SO_4$; II) Urea; III) Asparagine.

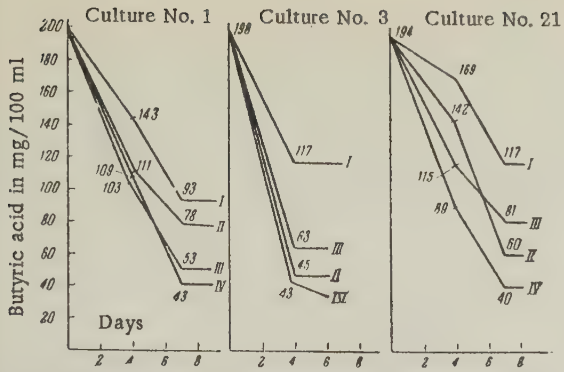


Fig. 3. Assimilation of butyric acid in the presence of I) 0.2% peptone; II) 1% peptone; III) 0.2% peptone + 0.01% $NH_4NO_3$; IV) 0.1% $NH_4NO_3$.

Table 1. Propagation of Active Butyric-Destroying Organisms in a Synthetic Medium with $NH_4NO_3$ and Peptone (in millions per ml of medium)

| No. of strain | 1 | | 3 | | 21 | |
|---|---|---|---|---|---|---|
| Duration of growth (in hr) | 120 | 240 | 96 | 192 | 96 | 192 |
| +0.1% $NH_4NO_3$ | 1293 | 960 | 520 | 560 | 1020 | 1910 |
| +0.2% Peptone | 688 | 1090 | 650 | 530 | 550 | 495 |

Table 2. Assimilation of Butyric Acid By Active Strains of Ammonificators in a Protein Medium (in ml 0.1 N $Ba(OH)_2$ per 100 ml of Medium)

| Strains | 1 | | | 3 | | 21 | | |
|---|---|---|---|---|---|---|---|---|
| Duration of growth (in hr) | 0 | 96 | 216 | 0 | 96 | 0 | 72 | 144 |
| Peptone water | 23.5 | 9.6 | 5.9 | 19.5 | 4.9 | 19.5 | 17.1 | 8.1 |
| Meat broth without peptone | 23.4 | 7.5 | 6.0 | 19.5 | 4.5 | 19.5 | 8.8 | 7.9 |
| MPB | 23.3 | 11.3 | 11.7 | 19.4 | 5.6 | 19.0 | 6.6 | 5.8 |

Particular attention was devoted to the elucidation of the effect produced by peptone. In view of the active destruction of acid in meat-peptone broth (MPB) and the slowness of the process in a synthetic medium we tested two dosages of peptone, 0.2 and 1%. In some tests we used the combination 0.2% peptone with 0.01% $NH_4NO_3$ in order to elucidate the significance of small concentrations of ammonium nitrogen. Fig. 3 shows that when 0.2% peptone was present the process was much slower and this was particularly pronounced in strain No. 21. In connection with this it should be noted that the ammonificating capacity of strain No. 21 was less pronounced than that of the other two. The process could be considerably accelerated by increasing the amount of peptone to 1%. The addition of 0.01% $NH_4NO_3$ to 0.2% peptone increased the energy of utilization of butyrate so that it approached that of the control.

A comparison of these findings clearly shows that the active assimilation of butyric acid is best secured by the presence of ammonium nitrogen in the medium. In their action on the speed of the process, the tested sources of nitrogen fall into the following order (starting with the most potent ones): salts > urea > asparagine > alanine > peptone > $KNO_3$. The slow rate of utilization of butyric acid when alanine, 0.2% peptone, and $KNO_3$ were present was probably due to the fact that the strains gave off ammonia while decomposing these substances. The correctness of this conclusion is borne out by the appearance in the medium of a distinct or intense reaction to ammonium at advanced stages of development of the culture. A further proof of the fact that, when a small amount (0.2%) of peptone is present, the assimilation of butyric acid takes place at the expense of its slow preliminary ammonification is the rapid and wholly satisfactory propagation of the strains in this variant (Table 1).

Only in the case of one variant, viz., when $KNO_3$ was present, ammonia was not observed in the medium. Evidently the ability to reduce nitrates to ammonia is acquired slowly by the strains according to their adaptability. The intensity of ammonification of the test nitrogen compounds corresponds to their accelerating action on the process.

In the light of these findings the effect produced by increased doses of peptone can be explained by the presence in a sterilized peptone of substances which contain ammonia either in a ready state or in an easily removable form. The high energy of the process in a meat-peptone broth can be explained in the same way. In order to determine the role of individual components of the given medium accurately, we investigated the course of assimilation of butyric acid in the following variants: (1) in peptone water (1%), (2) in a meat broth without peptone, and (3) in MPB. In these tests meat broth and MPB were prepared not with pure meat water but with a solution of nutritional broth cubes. Butyric acid was introduced in the form of 0.2% sodium butyrate. Analyses have shown that in all the variants butyric acid was actively consumed but it was difficult to determine the amount accurately because of the presence in acid distillations of small amounts of acetic acid and of a third unidentified high-molecular volatile acid. Table 2 gives some indication, if only very approximately, of the energy of the process. In this table the initial and residual content of volatile acids is expressed in milliliters 0.1 N $Ba(OH)_2$ used in the neutralization of 10 fractions of distillation. The strains multiplied intensively in all the protein media (Table 3).

It should be pointed out that when the above-mentioned media were sterilized but not sown with meat broth and MPB, they invariably gave a distinct positive reaction to ammonia. At advanced stages of growth of the strains, when the butyrate was already used up, the qualitative reaction to ammonia was more pronounced in meat media than in peptone water. Thus, the active consumption of butyric acid in MPB can be explained by the presence of certain amounts of ready ammonium nitrogen in it and by the subsequent ammonification of soluble albuminous nitrogen.

It is nevertheless necessary to emphasize the essential difference between the usual ammonification and the amonification produced by the isolated butyrate-destroying organisms. In the ordinary ammonificators the ammonia is the end product of disintegration of composite nitrous organic compounds used simultaneously as a source of both carbon and nitrogen. In the isolated microorganisms when butyrates are present in the medium, the physiological significance of ammonification rests in the production of

Table 3. Multiplication of Active Butyrate-Destroying Organisms in Protein Media with 0.2% Potassium Butyrate

| Strains | 1 | 3 | | 21 | |
|---|---|---|---|---|---|
| Duration of growth (hr) | 96 | 120 | 240 | 120 | 240 |
| Peptone water | 600 | 535 | 1465 | 835 | 1905 |
| Meat broth without peptone | 1500 | 565 | – | 2510 | 2930 |
| MPB | 1300 | 520 | 405 | 2300 | 2290 |

ammonium nitrogen, which is then used in the process of assimilation of butyric acid. In a similar way Mishustin (1936) explained the urolithic activity of two organisms which he had isolated and which showed a preference for ammonium nitrogen rather than any other nitrogen source. On this basis the organisms which we isolated should be regarded as a specialized group of ammonificators possessing a particular type of carbon nutrition. The specificity of this group of microorganisms is expressed in the fact that their energy of ammonification, as distinct from typical ammonificators, decreases as the molecules of the organic source of nitrogen became more complex.

It has already been demonstrated that ammonium nitrogen is essential for the proper assimilation of butyric acid and this is particularly evident in those synthetic media where the nitrogen is present in the form of a mixture of 0.2% peptone and 0.01% $NH_4NO_3$ (Fig. 3). In the literature there is similar information about the strong stimulating effect that small amounts of ammonium nitrogen have on the processes of oxidation of such acids as benzoic (Bernheim and de Turk, 1953), citric (Whitehouse et al., 1954), and oxalic (Jagnow, 1957). Whitehouse et al., (1954) demonstrated that yeast extract is necessary for the oxidation of citric acid because of the presence in it of small amounts of ammonia. The effect produced by such minute concentrations of ammonia supports the hypothesis that ammonia is essential for the fermentating apparatus which catalyses the oxidation of organic acids.

The ability of the isolated strains to endure and assimilate high concentrations of butyric salts can only occur and be strengthened in appropriate ecological conditions. If we take into account that the microorganisms described were isolated from dilutions of various kinds of soils (marshy, podzolized and hothouse soils) then there is sufficient ground to assume that at a certain definite stage of the conversion of the organic substance of the soil, conditions are created which result in the formation of considerable concentrations of butyric acid. The absence of factual information which would confirm this assumption can possibly be explained by the procedure employed. In the light of the data obtained it would be interesting to elucidate to what groups of soil microorganisms the specialized butyrate-destroying ammonificators belong and at what stage (or stages) of conversion of the organic substance of the soil the formation of large amounts of butyric acid salts can occur.

The acid-decomposing activity of microorganisms similar to those described in the present paper is one of the factors in the purification of the soil from butyric acid as a product of metabolism, the accumulation of which can have an adverse effect on the development of higher plants and representatives of soil microflora itself.

## SUMMARY


1. Intensive assimilation of butyric acid by a specialized group of ammonificators occurs when ammonium nitrogen is present in the medium.

2. When free ammonium nitrogen is absent, the energy of utilization of butyric acid depends on the ammonification rate of the nitrogen source present in the medium.

3. With regard to the accelerating effect upon butyrate utilization, the nitrogen sources can be arranged into the following series: ammonium salts > urea > asparagine > alanine > peptone > $KNO_3$.


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# THE LYSOGENICITY OF ACTINOMYCES ERYTHREUS CULTURES AND THE ISOLATION OF THEIR SPECIFIC ACTINOPHAGES

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Previously we reported on the isolation of two actinophages specific only for cultures of Actinomyces erythreus, which produces erythromycin (Rautenshtein and Retinskaya, 1960). Both of these actinophages (Nos. 3 and 121) were isolated when the filtrate of a suspension of podzol soil which was filtered after being kept in peptone-corn medium on a shaker for 48 hr was placed on a freshly prepared background of A. erythreus No. 8594.

Phage No. 3 was isolated from soil sample No. 3 when, simultaneously with the addition of the soil to the flask of peptone-corn medium, 2 ml of 24-hr mycelium of culture No. 8594 was also added (experiment with enrichment). Phage No. 121 was isolated from soil sample No. 121; in this case the experiment was set up without enrichment.

In these experiments, simultaneously with the A. erythreus No. 8594 culture, variants of this culture—A, B, C, and D, obtained from it through the combined action of UV and x-rays and ethyleneimine—were also used as test cultures.

We obtained A. erythreus No. 8594 and its variants, A, B, C, and D, from the Museum of Live Cultures and the Selection Laboratory of the All-Union Scientific Research Institute of Antibiotics. All of the variants of culture 8594 produced erythromycin and were sensitive to actinophages Nos. 3 and 121. However, when the same filtrates of soil suspension from which phages Nos. 3 and 121 were isolated on a background of A. erythreus No. 8594 were placed on backgrounds of these variants, phages could not be isolated.

The question of the origin of the isolated actinophages, Nos. 3 and 121, was not clear to us and we left it open in our 1960 experiment.

In the present work, we set ourselves the task of defining this question more precisely and, if possible, to determine whether actinophages Nos. 3 and 121 were actually present in the investigated samples of podzol soil, Nos. 3 and 121, or whether they were isolated from the culture of A. erythreus No. 8594 itself.

## EXPERIMENTAL PART

### Experiments on the Isolation of Actinophage from A. erythreus Cultures

Cultures of A. erythreus No. 8594 and its variants grow well at 33–34° C on peptone-corn medium (PCA), corn medium No. 6, and mineral medium No. 1. The composition of PCA (in %): glucose—1.0, peptone—0.5, corn extract—0.5, NaCl—0.5, $CaCO_3$—0.5, agar—1.5; pH—7.0.

The composition of corn medium No. 6 (in %): corn extract—0.5 (by dry weight), $CaCO_3$—0.3, $(NH_4)_2SO_4$—0.3, NaCl—0.3, starch—1.0, agar—2.0.

The composition of mineral medium No. 1 (in %): $MgSO_4 \cdot 7H_2O$—0.05, NaCl—0.05, $K_2HPO_4$—0.05, $KNO_3$—0.1, glucose—2.0, agar—2.0.

On corn medium strain No. 8594 forms round, flat, folded colonies, the color of the aerial mycelium is grayish, the submerged mycelium is dark brown, and the medium has a faint dark purple color. On mineral medium No. 1 strain No. 8594 forms folded colonies, the color of the aerial mycelium is grayish-pink, while that of the submerged mycelium is cherry with a brownish tint. The medium becomes a cherry-pink color.

When culture 8594 was inoculated as a thick background in petri dishes or in test tubes of slanted agar

Table 1. Results of Experiments on the Reisolation of Actinophages from Soil

| Soil sample and its characteristics | Storage time prior to reisolation | Experimental conditions and results | | | |
|---|---|---|---|---|---|
| | | without heating | | with heating | |
| | | without enrichment | with enrichment | without enrichment | with enrichment |
| No. 3, podzol, strongly fertilized with peat and manure, collected in the Botanical Gardens of Moscow State University | 3 months | – | – | – | – |
| | 4 " | – | – | – | – |
| No. 121, podzol, hothouse soil from the Main Botanical Garden of the Academy of Sciences, USSR | 14 days | – | + | – | – |
| | 1 month | – | – | – | – |

Note: + means that phage was isolated, – not isolated.

Table 2. The Presence in Soil Samples Nos. 3, 121, and 1527 of Actinomycetes Sensitive to Actinophages Nos. 3, 121, and 1527

| Soil sample No. | Number of actinomycetes isolated | | | Number of cultures which proved to be sensitive to the corresponding actinophage |
|---|---|---|---|---|
| | on medium without erythromycin | on medium with 10 units/ml of erythromycin | on medium with 50 units/ml of erythromycin | |
| 3 | 75 | 5 | 0 | 0 |
| 121 | 64 | 3 | 0 | 0 |
| 1527 | 62 | 0 | 0 | 0 |

medium, on PCA, corn, or other media rich in organic forms of nitrogen, large numbers of fine, almost pinpoint size sterile areas—negative colonies—frequently appeared on the growth surface of the culture (Figs. 1 and 2).

The appearance of such negative colonies was very rarely observed on mineral medium No. 1. The appearance of similar negative colonies can also be observed on a background of the variants of culture 8594—A, B, C, and D—but less frequently than in the original culture and in smaller amounts.

M. M. Meksina, who was studying the variability of the A. erythreus No. 8594 culture, also observed the appearance of lysed areas of the type of negative colonies on the surface of this culture when it was inoculated on agar slants; she reported this in the Bulletin of the All-Union Scientific Research Institute of Antibiotics, No. 5 (33), 1957.

The systematic appearance of negative colonies on the background of culture 8594 gave reason to consider it lysogenic. The following measures were therefore undertaken toward isolating the actinophage contained in this culture.

Small pieces of agar with a background of culture 8594 covered with negative colonies were transferred to flasks containing sterile broth. After repeated thorough shaking the liquid from the flask was centrifuged and then the presence in the centrifugate of phage particles capable of lysing culture 8594 and its variants A, B, C, and D on solid or liquid media was determined.

It was not possible to isolate such an actinophage by the indicated method. Culture 8594 was grown in peptone-corn medium on a shaker for 48 hr. The mycelium was then removed from the culture fluid by centrifugation and the presence in the centrifugate of phage particles capable of lysing and multiplying on culture 8594 and its variants in solid and liquid media was determined. In this case too such an actinophage could not be isolated from culture 8594.

Attempts to isolate actinophage by means of cross-testing culture 8594, its variants A, B, C, and D, as well as 50 strains isolated from single colonies after plating out culture 8594 also proved unsuccessful. The tests were carried out as follows: All experimental cultures were grown on liquid and agar-containing peptone-corn medium. The culture fluid from each culture was placed on backgrounds of all of the rest of the cultures and agar blocks of each 3-day-old culture were placed on freshly prepared backgrounds of all the rest of the cultures.

It is known that a lysogenic culture is resistant to the phage which it contains but may split off variants which do not contain the prophage and which therefore have become sensitive to the given symbiotic phage. Proceeding from this we plated out culture 8594 and isolated 500 cultures from separate colonies. All of these 500 cultures were used as indicators for sensitivity with respect to the actinophage which was assumed to be present in the culture fluid of strain 8594 after growth in peptone-corn medium on a shaker for 48 hr.

The experimental plates with cultures on whose backgrounds culture fluids had been placed were kept at 33–34° C for 96 hr and were examined daily. However, it was not possible to isolate actinophage from culture 8594 by this method either. When inoculated as a dense background on PCA small sterile zones—negative colonies—were also observed on the growth surface of all strains isolated when culture 8594 was plated out. Consequently they all continued to remain lysogenic and not a single nonlysogenic one was found among them.

Thus it was not possible to isolate an actinophage capable of lysing A. erythreus 8594 and its variants A, B, C, and D from these cultures by any of the methods we employed.

## Experiments on the Reisolation from Soil of Actinophages Specific for Erythromycin-Producing A. erythreus

The experiments on the reisolation of actinophages specific for A. erythreus were set up with the same samples of podzol soil—Nos. 3 and 121—which had been used for the isolation of actinophages Nos. 3 and 121; 210 samples of other soils were also used.

The same methods were used: 1) with enrichment by cultures 8594, A, B, C, and D and 2) without enrichment (Rautenshtein and Retinskaya, 1960).

Soil samples Nos. 3 and 121 were tested after various periods of storage under laboratory conditions. This was based on the data of preceding investigations (Rautenshtein and Kofanova, 1957), according to which actinophages remain in soil for a long time (not less than 10 months) when it is kept under laboratory conditions. Filtrates of soil suspensions obtained after keeping soil samples Nos. 3 and 121 in medium with and without enrichment on a shaker for 48 hr were placed on freshly prepared backgrounds of culture 8594, A, B, C, and D. Both unheated filtrates and filtrates heated for 10 min at 70° C were used in the experiments.

The results of these experiments are given in Table 1.

As seen from the data in Table 1, it was possible to reisolate actinophage specific for A. erythreus cultures in only one case, viz., when the unheated filtrate of soil No. 121 which had been stored under laboratory conditions for 14 days was placed on a background of culture 8594 in an experiment with enrichment. Since as has already been noted, actinophages usually remain in soil for quite a long time (up to 10 months) when it is kept under laboratory conditions, the data which we obtained place in doubt the question of whether actino-

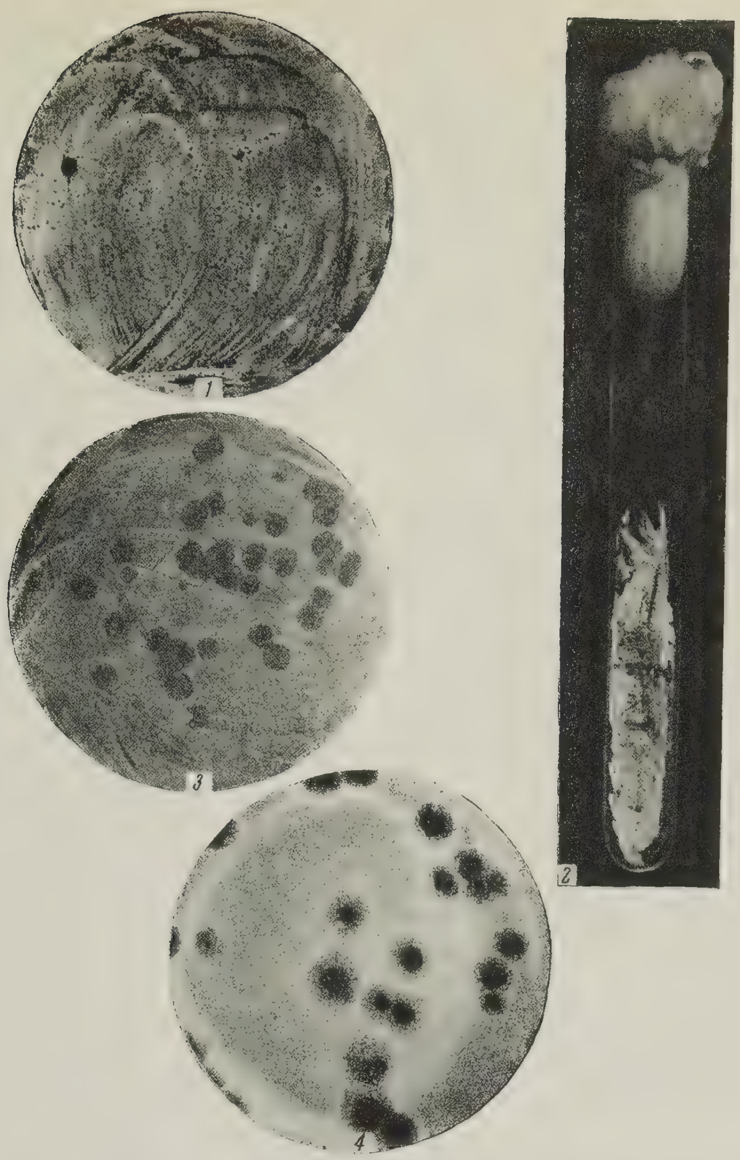

Fig. 1. Background of A. erythreus No. 8594 culture on corn-peptone medium in petri dish. Age 3 days; magnification 9:10.

Fig. 2. Growth of A. erythreus No. 8594 culture on peptone-corn medium in test tube. Age 4 days.

Fig. 3. Negative colonies of actinophage No. 1527 on background of culture No. 8594 on peptone-corn medium; single-layer method; magnification 8:10.

Fig. 4. Negative colonies of actinophage No. 121 on background of culture No. 8594 on peptone-corn medium; two-layer method; halos of inhibited growth are clearly visible around the negative colonies; magnification 8:10.

phages Nos. 3 and 121 existed at all in the soil samples under investigation. The reason for the failure to isolate actinophages from soil by the methods employed may have been low pH of the medium produced in individual cases when the soil was kept in nutrient medium on a shaker for 48 hr, as was shown by Khavina and Rautenshtein (1959). This possibility was excluded in the given case, however, because when soil sample No. 3 was tested, the pH of the culture fluid prior to filtration was within the range of 7.9–8.0 both in the experiment with enrichment and without enrichment, while in soil sample No. 121 the pH was 7.2–8.1.

In further experiments on the isolation from soil of actinophages specific for A. erythreus, 210 samples of various soils were used: from the Botanical Gardens of Moscow State University; the Main Botanical Garden of the Academy of Sciences, USSR; from under various tropical and subtropical plants; five fresh samples of chernozem soil from Kamennaya Steppe in the Voronezh Region; 37 samples of various soils from China, including yellow-brown soils and red soils; various hothouse soils; etc.

The methods with enrichment by culture 8594 and without enrichment were used. Culture 8594 and its variants A, B, C, and D were used as test cultures.

It was possible to isolate actinophage in only one case as the result of the tests done, viz., when the filtrate of soil sample No. 1527 was placed on a background of a culture of A. erythreus 8594 in an experiment without enrichment. Soil sample No. 1527 was collected in the subtropical areas of China in November of 1958 and was a moist chernozem with a pH of 6.7.

Experiments on the isolation of actinophages with this soil sample were set up after 8-month storage of this soil under laboratory conditions. The actinophage isolated from soil sample No. 1527, which we designated as actinophage No. 1527, proved to be very specific and entirely identical in its lytic property with the earlier isolated actinophages Nos. 3 and 121—it lysed only culture 8594 and all its variants.

As in the case of phages Nos. 3 and 121, Actinophage No. 1527 formed large negative colonies on A. erythreus cultures measuring up to 6–7 mm, surrounded by halos consisting of inhibited growth of the host culture (Figs. 3 and 4).

The latter is evidence of the fact that during lysis caused by this actinophage, as in the case of lysis caused by phages Nos. 3 and 121, lytic substances of the lysin type are present in the phage lysate. When phage No. 1527 acted on cultures sensitive to it profuse secondary growth consisting principally of phage-resistant forms appeared, as was the case when they were acted upon by phages Nos. 3 and 121.

A preliminary study of antigenic properties showed that serum obtained as the result of repeated immunizations of a rabbit with actinophage No. 121 completely inactivated actinophages 3 and 1527.

Phage-resistant variants obtained by treatment with phage 1527 were resistant to phages 3 and 121. Phage-resistant variants obtained by treatment with phages 3 and 121, in turn, were resistant to phage 1527, which also confirms their identity.

The Detection of the Presence in Soil Samples 3, 121, and 1527 of Actinomycetes Sensitive to Actinophages Nos. 3, 121, and 1527

In order to solve the problem of the origin of actinophages Nos. 3, 121, and 1527 it is very important to establish whether the corresponding soil samples contain actinomycete cultures sensitive to these phages on which they could multiply and consequently be preserved in the soil.

For this purpose all actinomycete cultures present in all three soil samples (Nos. 3, 121, and 1527) were isolated from them. The actinomycetes were isolated on the following media: synthetic No. 1, PCA, and PCA to which erythromycin was added (in a concentration of 12 units/ml in some experiments and 50 units/ml in others).

Preliminary experiments showed that the A. erythreus 8594 culture and its variants grow well on medium containing up to 200 units/ml of erythromycin and under these conditions are lysed well by their specific phages. Therefore the addition of erythromycin to the medium, which aids in the removal of actinomycete cultures sensitive to this antibiotic, should have facilitated the isolation of erythromycin-producing cultures. The latter, in connection with the specificity of actinophages 3, 121, and 1527, should have been sensitive to them.

The results of these experiments are listed in Table 2.

It is seen from the data in Table 2 that there were no actinomycete cultures sensitive to the corresponding actinophages in any of the soil samples tested. Consequently the conditions in these soils were not those required for the maintenance of these actinophages.

## DISCUSSION

In the problem of phage, the phenomenon drawing the greatest attention recently is that of lysogeny, which occurs widely not only among bacteria but among actinomycetes as well (Welsch, 1956; Shirling, 1956; Bradley, 1957; Rautenshtein, 1957a,b; Khavina and Rautenshtein, 1958). However, the isolation of actinophages, even from obviously lysogenic cultures, is often impossible due to the lack of appropriate indicator cultures.

It is well known that a truly lysogenic culture is usually resistant to the phage which it contains. The latter can be isolated if the lysogenic culture, spontaneously or with induction, releases phage for which there is an indicator culture.

Phage can also be isolated from a lysogenic culture in those cases where some particles of the symbiotic or temperate phage become virulent through the effect of various external and internal factors and acquire the ability to lyse the host culture. This explains the ability of some actinophages isolated from lysogenic cultures to lyse their host cultures, as described by some authors (Rautenshtein, 1957a,b; Khavina and Rautenshtein, 1958; Bradley, 1957).

In isolating actinophages from the soil and other substrates, it is quite possible that, when lysogenic strains are used as test cultures, the conversion of symbiotic (temperate) phage of the lysogenic culture to virulent phage occurs under the influence of the various substances contained in the soil filtrate. It is frequently quite difficult to establish exactly whether a given phage has been isolated from the substrate under investigation or whether it belongs to the lysogenic test culture. For example, Khavina and Rautenshtein (1958) isolated actinophage No. 8238 by placing the filtrate of a podzol soil suspension on a background of a museum type culture of A. olivaceus No. 8238. The phage isolated proved to be very specific. It is quite possible that this phage did not exist in the soil sample tested. However, in the filtrate of soil suspension placed on the background of the A. olivaceus 8238 culture there were substances causing the conversion of the temperate phage of this culture to virulent phage. Rautenshtein (1960) showed that the A. olivaceus 8238 culture is lysogenic and an actinophage which did not differ from actinophage No. 8238 in its spectrum of lytic activity was isolated from it. Aside from this, along with antibiotics (Rautenshtein, 1957a,b and 1960), phage lysate may also contain other substances capable not only of inducing the symbiotic phage contained

in the lysogenic test culture, but also of modifying the lytic activity of certain phage particles and converting them from temperate to virulent phage.

A similar type of phenomenon was observed by us and Khavina in some cultures of the A. lavendulae group.

The experimental data presented in the present work show that actinophages Nos. 3, 121, and 1527, isolated by the action of the filtrates of soil samples Nos. 3, 121, and 1527 on a background of an A. erythreus No. 8594 culture, were not contained in these soils. This is confirmed by: 1) the absence in the experimental soils of actinomycete cultures sensitive to these phages; 2) the consistent failure to reisolate the indicated phages from these soils; 3) the identity of all three actinophages isolated from different soils. The A. erythreus No. 8594 culture, as well as its variants A, B, C, and D, are undoubtedly lysogenic and are therefore resistant to the symbiotic actinophage which they contain. However, under the influence of certain substances arising when soil is kept in nutrient medium under certain as yet undetermined conditions, occasional particles of symbiotic phage are converted to virulent particles capable of lysing the host culture. Such, in our opinion, is the origin of actinophages Nos. 3, 121, and 1527.

## SUMMARY

1. When filtrates of two samples of podzol soil (Nos. 3 and 121) and one sample of red soil from China (No. 1527) were placed on a background of an Actinomyces erythreus 8594 culture (erythromycin producer), three actinophages—Nos. 3, 121, and 1527, specific only for erythromycin-producing A. erythreus cultures—were isolated.

Actinophage No. 3 was isolated in an experiment with enrichment, i.e., when a culture of A. erythreus 8594 was added simultaneously with soil to a flask of nutrient medium which was kept on a shaker 48 hr prior to filtration; phages 121 and 1527 were isolated in experiments without enrichment.

All three phages proved to be identical in lytic properties, in morphology of negative colonies, serologically, and according to cross-reactions for phage resistance.

2. It was established that all three of the actinophages isolated did not exist in the corresponding soil samples tested. This is confirmed by the complete absence in the experimental soil samples of actinomycete cultures sensitive to actinophages Nos. 3, 121, and 1527 and failure to consistently isolate the indicated phages from these soils.

3. It was shown that the A. erythreus 8594 culture is lysogenic and is resistant to the symbiotic phage which it contains.

4. Under the influence of certain substances existing in the filtrate and apparently arising in nutrient medium when soil is kept in it, individual particles of symbiotic phage from the A. erythreus 8594 culture are converted to virulent particles capable of lysing their lysogenic culture. Such, in our opinion, is the origin of actinophages Nos. 3, 121, and 1527, which are all identical.

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# A STUDY OF THE DEVELOPMENT OF ANTIFUNGAL ANTIBIOTIC-PRODUCING ACTINOMYCES

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Among antibiotic substances which inhibit the development of pathogenic fungi an important place is held by a group of so-called polyene antibiotics, many of which have been described in recent years (Tsyganov et al., 1959; Hazen and Brown, 1950; Lechevalier et al., 1953; Jajima, 1955).

These substances, produced by actinomycetes, include the chromoform group with double connections. According to the number of the latter, polyene antibiotics are divided into four types: tetraenes, pentaenes, hexaenes, and heptaenes (Orochnic et al., 1955). Some of them such as nisin, trichomycin, candicidin, and amphotericin B are being used in medicine; research is now being carried out on the utilization of polyene antibiotics in phytophathology. Nevertheless the chemical structure of these substances, the mechanism of their action, and the process of biosynthesis have so far been insufficiently investigated.

The objects of the present investigation were the identification – for the first time – of the antifungal substance produced by soil actinomycetes and the study of certain conditions of the formation of the antibiotic.

For the investigation we used actinomycetes isolated from rhizospheres of rust from soil near Moscow which had manifested a strong inhibiting action on the development of Candida albicans. The actinomycetes had a pale yellow, velvety, airy mycelium and a light brown substrate. Upon development it stained the medium a brown color. The spore carriers were either straight or slightly wavy, the spores were round. As the result of a detailed study of the morphological and culture symptoms the actinomycetes used in this work were identified as belonging to the Actinomyces globisporus type (Krasil'nikov, 1949); within this type, however, it could not be definitely identified with any of the known species.

When this microorganism was cultivated in a liquid medium in retorts on a rocking device, an antibiotic substance began to accumulate in both the culture liquid and the mycelium. From the culture medium the antibiotic was extracted with n-butanol in a ratio of 1 : 1. After distilling the solvent in a vacuum, a dry, greenish-brown amorphous powder remained which had an activity of 40,000 units/mg. The mycelium mass was washed, dried, and ground with quartz sand. For the extraction of the antibiotic, a 60% water ethanol was added. After separating out the solvent from the mycelium and evaporating in a vacuum, a powder similar to the one already described was left.

To study the antibacterial spectrum of the crude antibiotics, we used various test organisms. When tested on 20 different Gram-positive and Gram-negative microorganisms, the antibiotics did not inhibit their development, nor was there any inhibition of the development of actinomycetes. At the same time the isolated substances displayed a strong antifungal action. In large dilutions they inhibited the development of all the tested yeast and other similar organisms such as C. albicans, Saccharomyces cerevisiae, Torula utilis, Schizosaccharomyces pombe, Oidium lactis, Endomyces magnusii, Rhodotorula minuta. Fungicidal action was also observed on a number of cycelial fungi, among which were phytopathogenic: Aspergillus niger, Penicillium jantinellum, Fusarium solani, F. sporotrichella, Fusicladium dendriticum, Stemphylium alternarioides, Verticillium daniliae, Rhizopus nigricans, Neurospora citophila, Alternaria phaseolus, Cladosporium fulvum, Scolecotrichum.

To identify the isolated antibiotics we took spectra of their absorption of alcohol solutions in UV light, as it is already known that most of the antifungal substances have characteristic maximums of absorption under these conditions. Fig. 1 shows the spectra of absorption of substances under investigation when the latter are dissolved in methanol. Both substances,

Table 1. Values of $R_f$ When chromatographing the Substances Under Investigation in a Number of Solvent Systems

| Solvent systems | Raw antibiotic from the culture fluid | | Raw antibiotic from mycelium | |
|---|---|---|---|---|
| | first stain | second stain | first stain | second stain |
| Distilled water | 0 | – | 0 | – |
| $NH_4Cl$, 3% solution | 0 | – | 0 | – |
| Water saturated with butanol | 0.02 | – | 0.11 | – |
| Butanol saturated with water | 0.209 | 0.98 | 0.21 | 0.97 |
| Butanol-water-acetic acid (2 : 1 : 1) | – | 0.95 | – | 0.98 |
| Butanol-pyridine-water (1 : 0.6 : 1) | 0.62 | 0.94 | 0.63 | 0.98 |
| Benzol-water-acetic acid (2 : 1 : 2) | 0 | 0.99 | 0 | 0.99 |
| Water-acetone (1 : 1) | 0.06 | 0.94 | 0.05 | 0.96 |
| Butanol-methanol-water (4 : 1 : 1) | 0.38 | 0.94 | | |
| Benzol-methanol (4 : 1) | 0.28 | 0.98 | | |
| Chloroform | 0 | 0.94 | | |

Note. – indicates absence of stain.

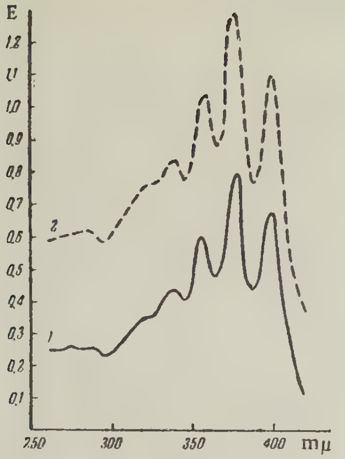


Fig. 1. Spectra of absorption of alcohol solutions of raw antibiotics in UV light. 1) From mycelium; from culture fluids.

i.e., those extracted from the mycelium and from the culture fluid, had identical spectra of absorption. Maximum absorption occurred in the regions 357-360, 375-380 and 400 mμ; this is characteristic of substances of the heptaene group of polyene antifungal antibiotics (Orochnic et al., 1955). The origin of two maxima at wavelengths of 255 and 340 mμ has not been elucidated; it may have been due to the presence of ballast substances.

Alcohol solutions of the antibiotics under investigation turned concentrated sulphuric acid blue, a phenomenon which is also characteristic of polyene compounds.

The data obtained regarding some of the properties of the isolated substances show that they are identical compounds belonging to the heptaene group.

To verify the nature of the distribution of the isolated antibiotics in several systems of solvents used for the study of new antibiotic substances and in particular of antibiotics with an antifungal action, we used the method of ascending chromatography on paper. The chromatograms were developed by the biological method on plates with a nutrient medium sown with C. albicans. The results presented in Table 1 show that the substances under investigation do not differ from each other even in the nature of their movement in various systems. Some of the magnitudes of $R_f$ are similar to those described in the literature for heptaene antibiotics. In a number of solvent systems the antibiotic divided into active fractions. A similar fission of certain heptaene antibiotics in a water-saturated butanol system as well as in butanol-pyridine-water (1 : 0.6 : 1) was described by Blinov in 1958. The substances under investigation also divided into two active components in such systems as benzol-water-acetic acid (2 : 1 : 2), benzol-methanol (4 : 1), water-acetone (1 : 1) and chloroform.

Peculiarities of the development of actinomycetes in conditions of submerged cultivation.

For the submerged cultivation of the product under investigation, we used as a base a synthetic medium of the following composition: $K_2HPO_4$ – 0.05%; $MgSO_4$ – 0.05%; NaCl – 0.05%; $FeSO_4$ – 0.005%; ordinary tap water; pH 7.0–7.2. After trying out this medium on a number of carbon-containing compounds and mineral sources of nitrogen we eliminated glucose and $KNO_3$. When actinomycetes were cultivated on a medium containing ammonium nitrogen $(NH_4)_2SO_4$, they developed well but had a very low antifungal action.

Table 2. Productivity of Mycelium of Actinomyces in Different Media

| Age of mycelium (in hrs) | Productivity | |
|---|---|---|
| | in a synthetic medium | in a synthetic medium with a Hottinger broth |
| 24–48 | 0.006 | 0.8 |
| 48–72 | 0.4 | 16.2 |
| 72–96 | 16.5 | 51.8 |
| 96–120 | 20.7 | 74.6 |

Note. Productivity was calculated by the formula: $(a_2-a_1)/(B_2-B_1)$: 2, where $a_2-a_1$ denotes the increase in activity for a definite interval of time and $B_2 + B_1$ denotes the difference of the biological mass for the same period.

When such complex organic substrates as peptone, Hottinger broth, yeast autolyzate or corn extract were added to the given medium there was a marked rise in the antibiotic activity and a slight increase in the biological mass of the mycelium.

We also investigated the development of the culture in a synthetic medium of the above-mentioned composition containing in one instance 2% glucose and 0.3% $KNO_3$ and in another variation 2% Hottinger broth.

Tests were carried out in conical retorts each containing 100 ml of medium. The retorts were placed on a rocking device. From specimens the weight of dry mycelium was determined; the residual nitrate nitrogen was determined calorimetrically, ammonium nitrogen by the cupric method, sugar by the Bari method, and pH – potentiometrically. The amount of antibiotic was determined after extraction with ethanol by serial dilutions separately in the culture fluid and in the mycelium and expressed in units of dilution per ml culture fluid. As test organism we used C. albicans.

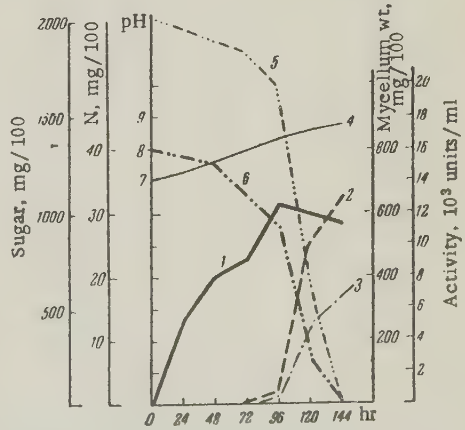


Fig. 2. Development of actinomycetes in a synthetic medium. 1) Weight of dry mycelium; 2) antibiotic activity of mycelium; 3) antibiotic activity of the culture fluid; 4) pH; 5) sugar 6) nitrate nitrogen.

The results of the tests are presented in the form of curves in Figs. 2-3. Actinomycetes developed in two stages: The antibiotic activity appeared after specific mutations had accumulated in the culture fluid and in the mycelium and reached its maximum value after attaining maximum biological mass. Both in a synthetic medium and in a medium with a Hottinger broth, the antibiotic began to accumulate in the mycelium earlier than in the culture fluid, then for a time the antibiotic activity increased at the same rate in both instances, after which the amount of antibiotic in the mycelium decreased while continuing to increase in the culture fluid. The distribution of total activity between the mycelium and the culture fluid was not constant and it was therefore impossible to determine it by any single age point of the growing culture. Synthesis of the antibiotic probably takes place inside the cells. The question of whether the liberation of the antibiotic into the medium represents an active process or whether it is associated with the autolytic disintegration of the mycelium remains unsolved.

A complex mixture of organic substances in the form of a Hottinger broth cannot replace either nitrate nitrogen or glucose. On the contrary, when the broth is used these substances are utilized much more actively. Moreover, the utilization of some of the broth substances by the developing culture considerably accelerates the whole process of development so that the second phase sets in sooner and more actively. In a synthetic medium, sugar and nitrogen are consumed over a longer period, the biological mass grows more slowly, and the level of antibiotic activity is low.

Of particular interest are the qualitative differences of the developing cultures in the given conditions, which show the amount of antibiotic produced per mg biological mass of mycelium for a given period of development. As the results presented in Table 2 show, the productivity of mycelium in a medium with a Hottinger broth considerably exceeds the productivity of mycelium on a synthetic medium.

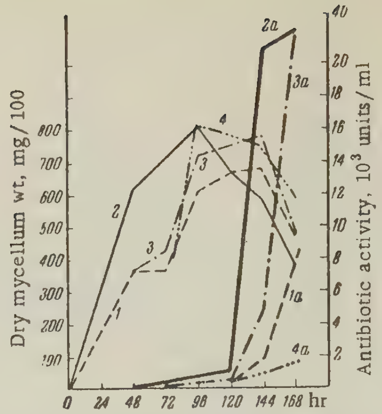


Fig. 4. Accumulation of biological mass and antibiotic activity on a synthetic medium when Hottinger broth was added at different times. 1,2,3,4) Biological mass; 1a, 2a, 3a, 4a) antibiotic activity; 1, 1a) on a medium without additions; 2, 2a) with 2% Hottinger broth; 3, 3a) with 2% Hottinger broth added after 48 hr; 4, 4a) 2% Hottinger broth added after 72 hr.

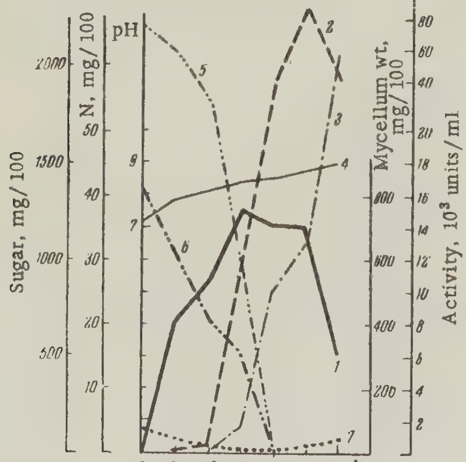


Fig. 3. Development of actinomycetes in a synthetic medium with a Hottinger broth. 1) Weight of dry mycelium; 2) antibiotic activity of mycelium; 3) antibiotic activity of the culture fluid; 4) pH; 5) sugar; 6) nitrate nitrogen; 7) ammonium nitrogen.

The introduction of Hottinger broth into the medium immediately after sowing, after 48 hr, and after 72 hr demonstrated that when it was introduced after 48 hr the development was very similar to if slightly slower than that observed in conditions when the Hottinger broth was introduced at the time of sowing. However, when an organic substrate was added 72 hr after sowing, a good growth of the biological mass was observed but the antibiotic activity was low (Fig. 4). Thus, for the biosynthesis of the antibiotic, despite the fact that it is formed in the second phase of development of the organism under investigation, it is desirable that a complex organic substrate be present in the medium at an earlier stage, since in the first phase of development conditions are already created which favor the formation of the antibiotic.

A microscopic examination of the morphology of mycelium during the development of actinomycetes the above-mentioned conditions revealed that there were two morphological cycles. During the first hours of development, which coincided with the first phase, spores began to germinate, the young mycelium grew and differentiated, and submerged spores also began to germinate, giving rise to new colonies. The young colonies were formed on the remains of the autolyzing primary mycelium, a part of which remained until the end of cultivation. This process coincided with the beginning of the appearance of antibiotic activity. The increase in the amount of antibiotic ran parallel to the increase and differentiation of the secondary mycelium.

Thus, the second physiological phase coincided with the second morphological cycle of development of the microorganism under investigation. When cultivated on a medium with a Hottinger broth or when the latter was added within 48 hr, an acceleration of the process was observed of all the above-mentioned morphological changes.

The authors wish to express their grateful thanks to Prof. V. N. Shaposhnikov for his guidance and help in this work.

## SUMMARY

1. A crude antifungal antibiotic was isolated from a soil actinomyces culture of the Actinomyces globisporus type. On the basis of its antibacterial spectrum and UV absorption spectrum it was found to belong to the heptane group of polyene antibiotics. According to the results of paper chromatography the antibiotic consisted of two active fractions.

2. The development of the actinomycete producer in submerged cultures occurred in two phases. The second phase, during which the antibiotic compound was formed, coincided with the germination of submerged spores with growth and differentiation of the secondary mycelium.

3. In the course of development of the actinomycetes, the antibiotic accumulated in the culture fluid and even earlier in the mycelium.

4. The addition of complex organic substrates, in particular the Hottinger broth, to synthetic media containing glucose and $KNO_3$ considerably accelerated the development of the organism under investigation and increased the amount of antibiotic produced.

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# INTERRELATIONSHIPS BETWEEN AZOTOBACTER AND TYPICAL RHIZOSPHERE BACTERIA OF CORN

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Indications are encountered in the literature citing both the positive effect of azotobacter on corn crops and the absence of any effect of this bacterial organism (Krasil'nikov, 1934; Blinkov, 1939; Meshkov, 1950; Zaremba and Sinyavskaya, 1954; Berezova, 1957; Gebgardt, 1953; Linchevskaya, 1953; Fedorov, 1944; and others). However, the reason for the diversity of the results obtained still remains unclear. It may be connected both with differences in the properties of the azotobacter strains used, and with their interrelationships with the typical rhizosphere bacteria of the given plant. Since in the course of our investigations, which were reported in the preceding communication (Fedorov and Savkina, 1960), we succeeded in isolating a number of typical rhizosphere bacteria of corn in pure cultures and studied their physiological peculiarities, it seemed interesting to ascertain the nature of their interrelationships with azotobacter as well. Accurate data on the nature of these interrelationships, even under conditions of laboratory culture, would be valuable material for resolving the problem of the causes leading to the inconsistent effect of inoculating corn seeds with azotobacter. With this consideration in mind, we undertook the present investigation.

Azotobacter chroococcum (strain 53), which is widely employed as the bacterial fertilizer azotobacterin, was used in these investigations. It was cultured on Fedorov's modification of nitrogen-free medium. Rhizosphere bacteria were introduced into the medium simultaneously with azotobacter in the form of a suspension of two-day-old agar cultures. The experiment was carried out in 100-ml conical flasks containing 30 ml of nutrient medium, and was continued for eight to ten days at 28-30°. At the end of the experiment, the number of azotobacter cells which had grown in the medium was determined, both in separate and in mixed culture with the rhizosphere bacteria of corn. The amount of atmospheric nitrogen fixed by them was also determined.

The results of these determinations are given in Table 1.

From these data it is easily established that, in the majority of cases, azotobacter in mixed cultures with the rhizosphere bacteria of corn fixed somewhat less atmospheric nitrogen than in separate cultures. However, the degree of inhibition of the given process did not exceed 15-20%. In two cases, the reverse phenomenon was even observed. When Chromobacterium flavum and Pseudomonas liquefaciens were present in the medium, the azotobacter fixed somewhat more atmospheric nitrogen, but here too the extent of activation was insignificant.

Table 1. The Effect of Typical Rhizosphere Bacteria of Corn on the Nitrogen-Fixing Activity of Azotobacter (0.614 g of glucose was utilized in each of the cultures)

| Experimental variants | No. of azotobacter cells grown, $10^6$ | Atmospheric $N_2$ fixed, in mg | | | |
|---|---|---|---|---|---|
| | | in separate culture | average | per g glucose utilized | % of control |
| Azotobacter chroococcum | 1102.4 | 7.50<br>7.20 | 7.35 | 11.90 | 100.0 |
| Same + C. flavum | 908.7 | 8.07<br>8.96 | 8.51 | 13.87 | 115.8 |
| Same + P. fluorescens | 763.3 | 6.75<br>6.14 | 6.44 | 10.49 | 87.7 |
| Same + B. agile | 893.7 | 5.61<br>6.14 | 5.87 | 9.56 | 79.8 |
| Same + P. radiobacter | 875.8 | 5.01<br>7.35 | 6.18 | 10.00 | 84.0 |
| Same + P. liquefaciens | 831.6 | 7.79<br>7.66 | 7.72 | 12.58 | 105.0 |
| Same + C. denitrificans | 861.0 | 7.19<br>6.49 | 6.84 | 11.15 | 93.0 |
| Same + C. aurantiacum | 437.4 | 7.60<br>6.11 | 6.85 | 11.16 | 93.2 |

Table 2. The Effect of Filtrates of the Rhizosphere Bacteria of Corn on the Growth of Azotobacter Cells and Their Nitrogen-Fixing Activity (average data of two repetitions)

| Experimental variants | Glucose consumed, mg | Atmospheric $N_2$ fixed per g glucose, mg | No. of azotobacter cells grown, $10^6$/ml |
|---|---|---|---|
| Azotobacter chroococcum | 577.0 | 6.83 | 854.3 |
| Same + C. flavum filtrate | 580.0 | 9.83 | 1226.2 |
| Same + P. fluorescens filtrate | 583.0 | 11.0 | 1821.4 |

In connection with this, it was interesting to deter-ine the reason for such an effect of the rhizosphere acteria of corn on azotobacter. It could have been onnected both with the partial utilization of glucose rom the nutrient medium by these bacteria, and with he secretion by them of substances inhibiting the rowth of azotobacter and its nitrogen-fixing activity.

In order to clarify the question of which of these ossible causes has the greatest significance, we onducted experiments in which filtrates of cultures f rhizosphere bacteria, grown separately from azo-obacter on Czapek's medium, were added to the utrient medium. For this purpose, cultures of rhizo-phere bacteria were grown for three weeks on Czapek's medium. The cells were then separated from he culture fluid by centrifuging at 3500 rpm, and the entrifugate obtained was filtered through a Chamber-and porcelain filter. The sterile filtrate of the culture f rhizosphere bacteria obtained in this manner was dded in 5-ml portions to sterile nitrogen-free med-um for azotobacter as modified by Fedorov. After his, the medium was inoculated with a two-day cul-ure of azotobacter and was incubated at 28-30° for 5 days. At the end of the experiment, the number of zotobacter cells grown was counted in the medium, nd the amount of atmospheric nitrogen fixed by them er g of glucose consumed was determined.

The results of these determinations are given in Table 2.

The effect of filtrates of cultures of the rhizosphere acteria of corn on the growth and nitrogen-fixing ctivity of azotobacter proved to be somewhat dif-erent from the effect of these bacteria during mixing ultivation with this microorganism. When filtrates ere added to the medium, both the number of azoto-acter cells growing up and their nitrogen-fixing ctivity increased markedly. This effect of the fil-rates indicates that, during mixed cultivation of hizosphere bacteria with azotobacter, not only were he rhizosphere bacteria activating azotobacter, but onsuming considerable glucose as well, thereby de-reasing the amount of energy-yielding material for he azotobacter and, consequently, its opportunity for rowth as well. This was most pronounced in cultures f P. fluorescens.

In mixed culture with azotobacter, this form clear-y inhibited nitrogen fixation, while its culture filtrate, n the contrary, strongly stimulated the given process. ince the intensification of nitrogen fixation was rought about through increased growth of azoto-acter cells, it must be assumed that the culture ltrates of these bacteria contain some supplementary growth factor which exerts a positive effect on the growth of azotobacter cells and on their efficiency in utilizing energy-yielding material. This hypothesis is confirmed by the following data: for the same amount of energy-yielding material (580 mg of glucose) utilized, 850 million cells grew in the medium without filtrate, while when 5 ml of filtrate was added, 1220-1820 million cells grew, near one and one-half to two times more. Since the added filtrates contained only traces of glucose and bound nitrogen, it must be concluded that these substances could not have had a noticeable effect on the increase in azotobacter growth. Evidently, the effect from the filtrates here could only have been obtained due to growth activators secreted by these bacteria into the media surrounding them. In connection with this, a new question arose. What is the nature of the activating effect of the filtrates, and on which enzyme systems of azotobacter do they have an activating effect? Toward this aim, we studied the effect of culture filtrates of the rhizosphere bacteria of corn on the respiration rate of washed azotobacter cells.

These investigations were carried out with the aid of Warburg's manometric method. Glucose (0.1 M), mannitol, and sodium acetate solutions were used as hydrogen donors. The azotobacter culture was grown on nitrogen-free agar medium and was used at the age of two days. Prior to the experiment, the cells washed off from the agar were washed with phosphate buffer at pH 6.98 and centrifuged. In testing each carbon source, the same number of cells were used, a suspension of which was added in equal volumes to the solution of hydrogen donor being tested; 0.5 ml of culture filtrates of the rhizosphere bacteria of corn was added to each Warburg vessel; 4.5 ml of a solution of the energy-yielding substance was added to the bacterial suspension, and respiratory activity was determined at 30°.

The results of these determinations are given in Table 3.

In all cases, culture filtrates of the rhizosphere bacteria of corn had a decided activating effect on the respiration rate of washed azotobacter cells. On glucose, the respiration rate increased seven to nine times in the presence of the filtrates, while on acetate, it increased one and one-half to two times in comparison with the control. Only on mannitol did the respiration rate remain at the level of the control when filtrates were added. Such a significant difference in the nature of the effects of the filtrates on the respiration of azotobacter, when using different hydrogen donors, indicates the dissimilarity of

Table 3. The Effect of Culture Filtrates of the Rhizosphere Bacteria of Corn on the Respiration Rate of Washed Azotobacter Cells

| Experimental variants | Amount $O_2$ consumed, $\mu l/5$ ml azotobacter cell suspension | | |
|---|---|---|---|
| | Hydrogen donor – glucose | Hydrogen donor – mannitol | Hydrogen donor – $CH_3COONa$ |
| Azotobacter chroococcum | 14.2, 13.2 } 13.70 | 39.25, 26.00 } 32.62 | 68.24, -- } 68.24 |
| Same + C. flavum filtrate | 93.10, 88.20 } 90.65 | 29.63, 42.00 } 35.81 | 86.50, 86.20 } 86.35 |
| Same + P. fluorescens filtrate | 107.05, 123.95 } 115.50 | 34.80, 36.70 } 35.75 | 107.5, 112.00 } 109.70 |

their influence on the different enzyme systems participating in this process. If the substances contained in these filtrates had a stimulating effect on the enzymes which activate molecular oxygen, then the same effect should have been obtained on all hydrogen donors. The considerable difference found in their activity on different hydrogen donors indicates that the basis of the effect is connected not with the cytochrome system and the hemin enzyme, but with dehydrogenases activating the hydrogen of the compounds being oxidized, or with the decarboxylases which bring about the splitting off of carbon dioxide from intermediate oxidation products (keto acids). Since the appropriate vitamins are required for the synthesis of both enzymes, the effect of the filtrates must apparently be connected with the presence of the corresponding vitamins in them. This hypothesis is confirmed not only by our data, but by the data of other investigators as well (Krasil'nikov, 1949) concerning the ability of P. fluorescens to synthesize an entire complex of vitamins. We obtained additional material confirming this hypothesis while studying the effect of the filtrates on the activity of dehydrogenases. Determinations showed that the activity of this group of enzymes was greatly increased under the influence of the filtrates. These experiments were carried out in evacuated Thunberg tubes to which methylene blue, washed azotobacter cells, and the appropriate 0.1 M hydrogen donor were previously added. In the presence of filtrates, the reduction of methylene blue proceeded rapidly, and was completed in 60-75 minutes, while without filtrate, it went on for 90 minutes. The greatest acceleration of reduction was observed under the influence of the P. fluorescens culture filtrate, which agrees with the data concerning the effect of this filtrate on the consumption of molecular oxygen. On this basis, it can be concluded that the basis of the activating effect of culture filtrates of the rhizosphere bacteria or corn is connected primarily with the effect of the vitamins they contain on the activity of dehydrogenases and, possibly, decarboxylases.

In connection with this, it must be particularly noted that the activation of these enzyme systems was also accompanied by the more efficient utilization of energy-yielding material for the processes of the synthesis of cell substance in azotobacter. This is suggested not only by the sharp increase in the total number of the cells grown per unit of energy-yielding material, but also by the sharp increase in their efficiency of assimilation of atmospheric nitrogen per g of carbon source utilized. This effect of the filtrates can only be explained by the more complete utilization of the energy of the oxidation of organic material, and it, in turn, is connected with its more complete accumulation in the form of high-energy phosphate bonds, which are evidently more easily formed in the presence of vitamins in the medium due to the more rapid synthesis of dehydrogenenases and decarboxylases participating in the mobilization and transfer of hydrogen from the oxidized substance to its final acceptor–molecular oxygen. Evidently, the greater the number of enzyme systems participating in this hydrogen transfer, the greater are the possibilities for fixing the energy released, because the energy level of the oxidizable hydrogen drops more slowly with less release of biologically useful energy at each stage of the oxidation. Due to this, conditions are created for the more complete fixation of the energy released in the form of high-energy phosphate bonds, which are further used for the vital processes of azotobacter. from these data, it must be concluded that, in the presence of the appropriate vitamins in the medium, azotobacter synthesizes a greater diversity of enzymes participating in oxidative processes and accumulates the energy of oxidation more completely in the cell protoplasm. This is what makes possible its more intensive growth, inevitably leading to increased efficiency of nitrogen fixation.

If the data on the effect of culture filtrates of the rhizosphere bacteria of corn on the growth and nitrogen-fixing activity of azotobacter are compared with the data on the inhibitory effect of these same bacteria on the growth and nitrogen-fixing activity of azotobacter under conditions of mixed culture, it must be concluded that their inhibitory effect in mixed cultures is chiefly associated with their utilization of part of the energy-yielding material from the substrate. Because of this, the azotobacter get less of the energy-yielding material and, in calculating the efficiency of nitrogen fixation per g of carbon source utilized without delimiting which part of it was used by the azotobacter and which part by the rhizosphere bacteria growing with it, a decrease will naturally be obtained. Aside from this, the possibility has not been excluded that, in mixed cultures with azotobacter, the rhizosphere bacteria of corn not only secrete vitamins into the medium, but antibiotic substances

as well. There are indications in the literature (Krasil'nikov, 1934) that a number of bacteria produce them at a high rate in mixed cultures, especially with competing microorganisms. Such a possibility is quite likely for P. fluorescens cultures, since the given bacterium is capable of active antibiotic production anyway. All this speaks for the complexity of the interrelationships between azotobacter and the rhizosphere bacteria of agricultural plants.

## SUMMARY

1. In mixed cultures with azotobacter, typical rhizosphere bacteria of corn noticeably inhibit its growth and nitrogen-fixing activity in the majority of cases. The only exceptions to this rule are Chromobacterium flavum and Pseudomonas fluorescens which, even in mixed cultures, increases the nitrogen-fixing activity of azotobacter somewhat. The inhibitory effect of rhizosphere bacteria is connected with their utilization of the energy-yielding material from the substrate.

2. Sterile filtrates of the cultures of the rhizosphere bacteria of corn which we investigated, including those which inhibit the growth of azotobacter in mixed culture, definitely stimulate its growth and nitrogen-fixing activity. The stimulation is associated with the presence in these filtrates of supplementary growth factors which activate the respiration of azotobacter. Since the activation differs markedly on different hydrogen donors, it must be assumed that it is connected only with the activation of dehydrogenases and possibly of decarboxylases. These activators have no effect on the cytochrome system and the hemin enzyme.

3. The activation of dehydrogenases is apparently associated with the presence in the filtrates of vitamins used by the azotobacter for the synthesis of these enzymes. Due to their rapid synthesis and, possibly, to their greater diversity, the efficiency of the utilization of oxidation energy for the vital processes of azotobacter is increased, leading to increased growth and greater efficiency of nitrogen fixation.

4. The interrelationships between azotobacter and the rhizosphere bacteria of agricultural plants are complex and varied, and depend on the conditions of cultivation of these microorganisms.

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# THE EFFECT OF THE COTTON PLANT ON THE GROWTH OF AZOTOBACTER IN SOIL

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There is contradictory information concerning the effect of cotton plant cultures on the growth of azotobacter in soil.

According to Krasil'nikov's data (1945, 1958), the root system of the cotton plant inhibits azotobacter. Under field conditions, in crop rotation (lucerne-cotton) the amount of azotobacter in the soil decreases under cotton and increases under lucerne. Analogous data were also given by Kuzina (1955).

However, the majority of authors – Kononova (1929), Raznitsyna (1947), Kvasnikov and Petrushenko (1955), Lazarev (1954, 1957), Mishustin and Naumova (1954), and Kiseleva et al. (1958) – reported good growth of azotobacter in soil under cotton which, according to some data, was not exceeded by the growth of azotobacter under lucerne. Kvasnikov and Petrushenko (1951) and Lazarev (1957) observed no inhibition of azotobacter under cotton.

In view of the importance of this question in studying the fertility of the irrigated soil of cotton-raising regions, in the present work, we attempted to study the chief factors determining the growth of azotobacter in soil under cotton. Moreover, we proceeded from the fact that, for the correct evaluation of the effect of the cultivated plant on the soil microflora, it is necessary to analyze not some one factor (rhizospheres), but a complex of conditions occurring together during the growth of this culture: soil-climatic conditions required for the given plant, the composition and conditions of decomposing of root and harvest residues, the composition of the rhizosphere, procedures for cultivating the plants (agrotechnology), etc.

The work was carried out in 1954-1958 in the fields of the Iolotan' and Mary Experimental Station (Murgab Oasis, Turkmen SSR). The soils investigated belong to the desert, dry lake bed, moderately argillaceous type with very slight amounts of humus (0.6-0.8%) and total nitrogen (0.06-0.09%). The soils are long-irrigated, nonsaline or slightly saline, and well cultivated, and give high yields of cotton and lucerne.

## METHODS

Samples for microbiological analysis were taken with a borer from five to ten points in the plot. As a rule, the analyses were carried out the day that the samples were collected. Azotobacter was counted by the agar plate method on medium of the following composition (in g): sucrose – 10.0, $K_2HPO_4$ – 0.5, $KH_2PO_4$ – 0.5, $MgSO_4 \cdot 7H_2O$ – 0.2, $CaCO_3$ – 5.0, leached agar – 20.0, irrigation water – 100 ml. Deep inoculations were made; the height of the medium layer was 3-4 mm. The medium and method of inoculation were recommended as standard by the All-Union Conference on Soil Microbiology in Leningrad (1953). Counts were made on four parallel Petri plates on the fourth and seventh or eighth days. The deep method of inoculation in shallow layers of medium does not inhibit the growth of azotobacter colonies and does not interfere with their recognition. At the same time it facilitates the counting of colonies considerably since they do not coalesce.

The following method of sample collection and analysis was employed in analyzing the rhizosphere of cotton.

Small cuts in the root system were made to a depth of 20-25 cm near clusters of cotton plants at five to seven points in the plot.

Table 1. Azotobacter and Olingonitrophiles in the Soil and on the Roots of Cotton (per g of absolute dry soil and roots)

| Date of analysis | Stage of development of cotton plant | Azotobacter (thousands) | | | | | | Oligonitrophiles (millions)* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | soil | | roots | | | | soil | | roots | | | |
| | | between clusters | near roots | 1st wash | 2nd wash | 3rd wash | washed and ground | between clusters | near roots | 1st wash | 2nd wash | 3rd wash | washed and ground |
| 11.V | Two true leaves | 4.7 | 7.0 | 2.4 | 0.1 | 0.05 | 0.00 | 15.9 | 20.6 | 2430 | 372 | 157 | 34[illegible] |
| 21.VI | Budding | 7.7 | 3.8 | 0.5 | 0.15 | 0.02 | 0.00 | 16.6 | 16.2 | 510 | 121 | 66 5 | 14[illegible] |
| 23.VII | Blossoming and boll formation | 5.3 | 6.0 | 0.6 | 0.2 | 0.04 | 0.05 | 8.9 | 10.4 | 376 | 64.7 | 60.5 | 17[illegible] |
| 11.X | Ripening | 2.7 | 4.6 | 2.4 | 0.4 | 0.2 | 0.03 | 9.1 | 11.8 | 81 | 35.4 | 24.0 | 89.[illegible] |

* Counts on medium for azotobacter as modified by M. V. Fedorov.

Table 2. Comparative Numbers of Azotobacter in Soil from Old Cotton Plowland and under a Second-Year stand of Lucerne (in thousands per g of absolutely dry soil from the 0-40 cm horizon)

| Date of analysis | 1954, 6th year cotton | | | | | |
|---|---|---|---|---|---|---|
| | azotobacter cells | | | soil moisture, % | | |
| | fertilizers | | | fertilizers | | |
| | O | NK | NPK | O | NK | NPK |
| 16—17.VII | 9.5 | 4.6 | 6.9 | 15.5 | 12.0 | 15.1 |
| 20—21.IX | 11.3 | 8.8 | 16.2 | 16.9 | 14.9 | 15 2 |
| 3—6.XI | 8.1 | 4.2 | 6.7 | 7.5 | 3.4 | 5.1 |
| Average | 9.6 | 5.9 | 9.9 | | | |

| Date of analysis | 1956, 2nd year lucerne | | | | | |
|---|---|---|---|---|---|---|
| | azotobacter cells | | | soil moisture, % | | |
| | fertilizers* | | | fertilizers* | | |
| | O | NK | NPK | O | NK | NPK |
| 22.VIII | 6.0 | 5.5 | 4.1 | 10.8 | 10.7 | 12.7 |
| 26.IX | 5.4 | 3.0 | 4.0 | 12.6 | 9.3 | 10.4 |
| 15.XI | 4.1 | 3.5 | 4.5 | 14.8 | 13.2 | 15.6 |
| Average | 5.2 | 5.2 | 4.0 | 4.2 | | |

*Fertilizers were added under cotton. In six years, the following were added: N – 535 kg/ha; $P_2O_5$ – 300 kg/ha; $K_2O$ – 125 kg/ha.

The following were taken for analysis: 1) soil from between clusters, 20-25 cm from the main roots at a depth of 3-5 to 12-18 cm; 2) soil from a zone with a radius of 5-8 cm around the main roots at the same depth; 3) the roots themselves: healthy roots with a diameter of not more than 1 mm were cut off with scissors; in the early period of development of the cotton plant (May), plant roots were taken in entirety.

The soil was analyzed by the usual method. Large clumps of soil were removed from the roots. An average sample of 1-2 g of roots was taken for analysis. A weighed portion was placed in a flask, 100 ml of sterile irrigation water was poured over it, and it was shaken for 5 minutes. The suspension was analyzed as the first root wash. The water was then poured off from the flask with the aid of a sterile pipette. The roots and flask were washed slightly with 10 ml of sterile water. This water was discarded. The roots were again flooded with 100 ml of water from a pipette and shaken for five minutes. This was the second wash. The third, and sometimes the fourth washes were obtained in exactly the same manner. After the third or fourth wash, the roots were ground for three minutes in a mortar with sterile sand. The ground slurry was transferred to a flask with 100 ml of water and shaken for five minutes. A suspension of washed and ground roots was obtained. After appropriate dilutions, the washes were used for inoculating the media.

Laboratory experiments to ascertain the suitability of various plant residues for the growth of azotobacter were carried out in glasses with 200 g of soil taken from a plot of old cotton plowland which had been fertilized with complete mineral fertilizer. The plant residues were used in the ground form. Incubation was carried out at room temperature (26-34°) and about 60% of the total moisture capacity of the soil. The experiments were repeated 3-4 times.

## Azotobacter in the Rhizosphere of Cotton Plants

We studied the distribution of azotobacter in the soil near the root system of cotton plants and on the roots themselves over a period of two seasons. Similar results were obtained in both cases. Data for 1956 pertaining to an unfertilized plot are given in Table 1. The application of complete $N_{75}P_{50}K_{25}$ fertilizer did not change the character of distribution of azotobacter on cotton plant roots.

Investigation showed that there were somewhat more azotobacter cells in the soil taken within a radius of 5-8 cm from the main roots ("near roots") than in the soil between clusters. This can be explained by the greater content of food for azotobacter in the form of dead root particles near the roots, and by better aeration. On cotton roots themselves, we found considerably fewer azotobacter cells than in the soil both close to and at a distance from the roots. There was particularly little azotobacter here during the periods of budding and blossoming of the cotton. There was practically no azotobacter in immediate proximity to the roots (last wash, washed and ground roots). Consequently, the root system of growing cotton does not attract or concentrate azotobacter cells on itself.

One of the possible reasons for this is the profuse growth of oligonitrophilic microorganisms on the roots of cotton (Table 1). Growing well where there is a deficiency of nitrogen, oligonitrophiles are apparently the closest competitors of azotobacter in the struggle for sources of carbon nutrients under natural soil conditions.

## Azotobacter in Soil

According to the data of numerous analyses, azotobacter was found in considerable quantities in the soil of the fields investigated both under cotton and under lucerne. The number of azotobacter cells recorded usually fluctuated from several thousands to 10-20 thousand per g of soil from the plowed horizon (0-30-35 cm). Prolonged three and six-year cultivation of cotton in the crop rotation (three years of grasses – six years of cotton) did not lower the numbers of azotobacter in the soil. Thus, at the Iolotan' Experimental Station, in a field where cotton was grown the first year after plowing the grass layer, the amount of azotobacter fluctuated from 3500 to 8900 cells per g of soil from the plowed horizon; in a field with the second year of cotton, it fluctuated from 3900 to 10,000; in a field with the third year of cotton – from 3800 to 10,600; and in a field with the sixth year of cotton – from 8100 to 13,200, respectively (the data pertains to unfertilized plots). Under lucerne, especially under two and three-year lucerne, azotobacter was encountered in noticeably smaller numbers than under cotton. Special observations were made on one of the fields where, in 1954, cotton was grown for the sixth year and in 1956, on the second year of growing

Table 3. Amount of Azotobacter in Various Crop-Rotation Fields in the Plowed and Subplowed Horizons

| Horizon, in cm | Crop-rotation field | | | | | Crop-rotation field | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Lucerne | | Cotton | | | Lucerne | | Cotton | | |
| | first year | third year | first year | second year | sixth year | first year | third year | first year | second year | sixth year |
| | Azotobacter cells in thousands per g of absolutely dry soil | | | | | Soil moisture, in % | | | | |
| 0–30 | 5.2 | 2.0 | 7.0 | 15.0 | 7.1 | 10.8 | 14.6 | 4.9 | 10.8 | 7.9 |
| 30–40 | 2.3 | 0.4 | 0.6 | 2.2 | 4.9 | 14.9 | 11.2 | 6.1 | 8.8 | 11.6 |

Note. Counts were made on mannitol-containing medium.

Table 4. The Growth of Azotobacter When 1% of Organic Material was Added to the Soil

| Time of composting, days | Azotobacter cells, $10^3$/g absolutely dry soil | | | | | |
|---|---|---|---|---|---|---|
| | control | cotton roots | cotton leaves | lucerne roots | half-rotted manure | sucrose |
| 0 | 12.5 | 12.5 | 12.5 | 12.5 | 12.5 | 12.5 |
| 2 | 11.2 | 813 | 686 | 52.7 | 12.3 | 278 000 |
| 6 | 11.1 | 588 | 380 | 39.8 | 11.8 | 246 000 |
| 34 | 10.7 | 190 | 344 | 15.4 | 9.0 | 53 900 |
| 60 | 9.5 | 102 | 234 | 11.9 | 10.0 | 28 600 |
| Average | 10.6 | 423 | 411 | 29.8 | 10.8 | 151 600 |
| Increase in comparison to control | 1.0 | 39.9 | 38.8 | 2.8 | 1.02 | 14 302 |

grasses. These observations were made on the same plots and at approximately the same intervals of time (a certain difference is explained by different times of watering the grasses and cotton).

The two-year stand of lucerne (Table 2) decreased the amount of azotobacter in the soil. This was more pronounced on an unfertilized plot and where complete NPK fertilizer was added under cotton. In the plot previously fertilized only with nitrogen-potassium fertilizer, through the action of which azotobacter was inhibited, the difference between the soil under cotton and under grasses (lucerne) was less significant.

It is also important to note that cotton, in distinction from lucerne, apparently promotes the enrichment of the subplowed horizon of soil with azotobacter cells (Table 3).

Root and harvest residues of cotton are apparently sources of carbon nutrients for azotobacter in cotton fields. Semergei (1953) observed considerable nitrogen fixation when 1% cotton root was added to the soil. As much as 10-16 mg of atmospheric nitrogen was fixed per g of root. According to Petrosyan (1940), azotobacter can grow at the expense of cotton husks and cottonseed cakes.

A special laboratory experiment showed that the root and harvest residues of cotton (principal mass of the latter–leaves) added to the soil increase the amount of azotobacter in it tens of times (Table 4).

The addition of lucerne roots also increased the amount of azotobacter, but to a lesser extent, which is apparently connected with their higher nitrogen content – to 2.95% (Bolotnikova, 1938) – as compared with cotton roots – 0.5-0.9% (Semergei, 1953).

## The Effect of Agrotechnical Factors

An important factor determining the character of the effect of the cultivated plant on one or another group of soil microorganisms is the method of cultivation, or agrotechnology of this culture.

Table 5. The Effect of Basic Plowing on the Growth of Azotobacter in Soil

| Method of working the soil | Horizon, in cm | Azotobacter cells, $10^3$/g absolutely dry soil | |
|---|---|---|---|
| | | March-May (average of 7 analyses) | June-October (average of 7 analyses) |
| Plowing to 30 cm with turning of layer | 0—10 | 7.1 | 4.6 |
| | 10—30 | 9.9 | 6.9 |
| Without plowing | 0—10 | 4.2 | 8.4 |
| | 10—30 | 2.8 | 3.6 |

(I. D. Shubin's field experiment)

Such agrotechnical procedures as basic plowing (Table 5), waterings, and individual between-row cultivation promote the development of azotobacter in soil under cotton plants.

Phosphorus fertilizers stimulated the growth of azotobacter in soil under cotton (Table 6, compare variants NK and NPK). However, the application of mineral nitrogen-potassium fertilizers alone (with doses of nitrogen of about 100 kg/ha annually) reduced the amount of azotobacter in the soil by about one and on-half to two times, although it did not cause its severe inhibition in these dosages (Table 6).

## General Evaluation of the Conditions for Growth of Azotobacter Under Cotton

Long-irrigated desert soils of the Murgab Valley are favorable for the growth of azotobacter, and the potential possibilities for its development here are exceptionally great. When 0.5-1.0% of easily available carbon source (sucrose) is added to the soil and there is an adequate supply of phosphorus, the amount of azotobacter reaches several hundred million cells

Table 6. The Effect of Mineral Fertilizers on the Amount of Azotobacter in Soil (Iolotan' Experimental Station: K. I. Semergei's experiments with fertilizers over a period of many years)

| Crop-rotation field | Doses of fertilizer, kg/ha | Date of sample collection | Horizon, cm | Azotobacter cells, $10^3$/g absolutely dry soil | | |
|---|---|---|---|---|---|---|
| | | | | O | NK | NPK |
| Third year – cotton | For year of study | 24.III | 0—43 | 3.1 | 2.4 | 3.3 |
| | N —100 | 11.V | 0—43 | 8.7 | 5.2 | 11.8 |
| | $P_2O_5$ — 50 | 19.VII | 0—43 | 8.2 | 6.2 | 9.7 |
| | $K_2O$ — 25 | | | | | |
| | In 3 years | | | | | |
| | N —150 | 6.X | 0—43 | 9.0 | 4.4 | 10.4 |
| | $P_2O_5$—145 | | | | | |
| | $K_2O$ — 50 | 13—14.XII | 0—43 | 3.3 | 3.1 | 3.3 |
| Sixth year – cotton | For year of study | 16—17.VII | 0—40 | 9.5 | 4.6 | 6.9 |
| | N —105 | 20—21.XI | 0—40 | 11.3 | 8.8 | 16.2 |
| | $P_2O_5$ — 50 | | | | | |
| | $K_2O$ — 25 | 3—6.XI | 0—40 | 8.1 | 4.2 | 6.7 |
| | In 6 years | | | | | |
| | N — 535 | | | | | |
| | $P_2O_5$—300 | 11—12.II | 0—40 | 7.3 | 3.7 | 6.1 |
| | $K_2O$ —125 | | | | | |

Note. Nitrogen was applied in the form of ammonium nitrate, phosphorus – in the form of superphosphate, potassium – in the form of potassium chloride.

per g of soil as early as the second day of the experiment.

In soils under grasses (lucerne), conditions are less favorable for azotobacter than under cotton, especially in the subplowed horizon. This is explained by the small doses of phosphorus fertilizers, the impossibility of embedding them deeply, lack of cultivation of the soil, and by the fact that the root residues of lucerne are less available to azotobacter as a source of nutrients.

The negative factor for azotobacter in cotton fields is not the cotton culture itself, but the very considerable doses of nitrogenous mineral fertilizers added to the soil under this culture. However, a number of factors apparently moderate the harmful effect of mineral nitrogen on azotobacter in the soils investigated.

## SUMMARY

1. General soil and climatic conditions in the desert dry lake bed soils of the Murgab Oasis of the Turkmen SSR which have been irrigated over a long period of time favor the growth of azotobacter.

2. Long (6 year) cultivation of a cotton-lucerne crop rotation does not inhibit the development of azotobacter in the soil. Under the cotton plant, azotobacter is found in larger quantities than under 2-3 year lucerne.

3. Considerably fewer azotobacter cells were found on the roots of growing cotton plants than in the soil.

4. The roots and other residues of the cotton plant are assimilated well by azotobacter. Lucerne roots are a less suitable source of nutrients for azotobacter.

5. Plowing, watering, and cultivation stimulate the growth of azotobacter in the soil under cotton. The agrotechnology for lucerne is less favorable for the growth of azotobacter.

6. Mineral nitrogen-potassium fertilizers (at doses of nitrogen ca. 100 kg per ha every year) can substantially (but not drastically) reduce the azotobacter content of the soil. Phosphorus fertilizers, even with a background of nitrogen-potassium ones, favor an increase in the azotobacter content of the soil.

7. Cotton should not be regarded as a culture which inhibits azotobacter in the soils investigated.

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# THE HYDROLOGICAL STRUCTURE OF THE ATLANTIC OCEAN AND THE NORWEGIAN AND GREENLAND SEAS ACCORDING TO MICROBIOLOGICAL DATA

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Deep-sea microbiological investigations in the open part of the Atlantic Ocean were conducted by Certes (1884), Fischer (1894), Chun (1899), Otto and Neumann (1904), Gazert (1912), and Levin (1899) in the Greenland Sea. These investigations showed that the content, in the water, of heterotrophic microorganisms which grow on protein media varies over a wide range depending on depth and geographical location.

In order to characterize the distribution of the microbial population of the water mass of the Atlantic Ocean and the adjoining Norwegian and Greenland Seas more completely, we carried out a program of investigations providing for the collection of water samples, from the surface to the bottom, at all hydrological stations during expeditions on the "Obi" (1956) in the Greenland Sea, on the "Sevastopol" (1958) in the Norwegian Sea and on the "M. Lomonosov" (1959) in the Atlantic Ocean.

In the Atlantic Ocean, investigations were carried out along the 30th meridian at 41 stations from 66° north latitude to the Tropic of Capricorn. In the Norwegian Sea, four sections with 51 stations were made from the Scandinavian Peninsula in the direction of Iceland and the Island of Jan Mayen. In the Greenland Sea, 34 microbiological stations were situated primarily on three latitudinal sections at 78°, 79°, and 80° north latitude (Fig. 1). Microbiological analysis of the surface ground layer was carried out at six stations in the Atlantic Ocean, eight stations in the Norwegian Sea, and seven stations in the Greenland Sea. The samples collected were argillaceous or sandy silt with an admixture of foraminifera; some had a brown or brownish-grey color.

## METHODS

The water samples for microbiological analysis were collected with a Nansen-type bathometer from the following standard horizons at each station: 0, 10, 25, 50, 75, 100, 150, 200, 250, 300, 400, 500, 600, 750 (or 800), 1000, 1500, 2000, 2500, 3000, and deeper after each thousand meters; in particular cases, samples were taken at intermediate horizons. The water samples, collected with all microbiological precautions, were immediately examined by the germination on membrane ultrafilters method (Kriss, 1959) in microbiological laboratories installed on the ships. For filtration, 40 ml of water was taken. After filtration of water samples, the No. 2 membrane ultrafilters were placed with their reverse sides on the surface of nutrient agar (40% agar and 60% tryptic hydrolyzate of fish meal) for the germination of the microbial cells which had settled on the filter. Ten consecutive dilutions of silt samples were inoculated into nutrient broth (0.5 ml of the given dilution, and 0.25 ml of a 1 : 10 silt dilution on nutrient agar). The nutrient media were prepared with sea water. Incubation was carried out for 4-7 days at 18-35°; then, the number of colonies growing on the membrane ultrafilters was counted, and representatives of different kinds of colonies were subcultured on agar slants of the same composition.

A total of 1733 water samples were examined in this manner.

## EXPERIMENTAL RESULTS

As was the case in the Antarctic Ocean (Kriss, Lebedeva, Abyzov, and Mitskevich, 1958), in the high latitudes of the Atlantic Ocean, the entire water mass with the exception of specific layers is very poor in heterotrophic microorganisms. From the majority of samples, single colonies grew on the membrane filters after 40 ml of water was filtered through them, or else the filters remained sterile. This picture was observed at stations between 70° and 40° N (Fig. 2).*

In the subtropical region (40-23° N), the content of heterotrophs in the water mass of the ocean begins to increase and, analogously to what was found in the Pacific and Indian Oceans (Kriss, Lebedeva, Abyzov, and Mitskevich, 1958), reaches the very highest concentrations in the equatorial-tropical zone. In this zone of the Atlantic Ocean, water samples collected from the surface to the demersal layers contained, with the exception of certain horizons, such a considerable number of heterotrophic microorganisms that hundreds of colonies grew on the filters (Fig. 3).

*In this and similar figures, in all cases when not a single colony (0) grew from 40-50 ml of water from the given sample, the points were placed directly on the ordinate axis in order to avoid the complications of curves ($\log 0 = -\infty$).

Density of Microbial Population (Heterotrophs) in Various Geographic Zones of the World Ocean

| Lat. | Pacific Ocean | | | | Indian Ocean | | | | Atlantic Ocean | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | no. of samples | no. of colonies on filters | | | no. of samples | no. of colonies on filters | | | no. of samples | no. of colonies on filters | | |
| | | 0–9 | 10–99 | over 100 | | 0–9 | 10–99 | over 100 | | 0–9 | 10–99 | over 100 |
| 76–60°N | | | | | | | | | 71 | 95.7 | 4.3 | 0 |
| 60–50°N | | | | | | | | | 78 | 93.7 | 1.3 | 0 |
| 50–40°N | | | | | | | | | 83 | 90.0 | 10.0 | 0 |
| 40–23°N | 152 | 44.4 | 32.5 | 23.1 | | | | | 104 | 44.3 | 38.4 | 17.3 |
| 23–10°N | 165 | 13.4 | 41.2 | 40.4 | 74 | 1.4 | 5.4 | 93.2 | 121 | 1.6 | 33.9 | 64.5 |
| 10N–10°S | 278 | 16.6 | 31.3 | 52.1 | 106 | 7.5 | 3.8 | 83.7 | 167 | 3.0 | 20.0 | 77.0 |
| 10–23°S | 160 | 24.7 | 48.7 | 26.6 | 89 | 11.2 | 14.6 | 74.2 | 81 | 2.5 | 20.0 | 77.5 |
| 23–40°S | 272 | 37.6 | 48.5 | 13.9 | 176 | 42.0 | 31.2 | 26.8 | | | | |
| 40–50°S | | | | | 135 | 61.5 | 16.3 | 22.2 | | | | |
| 50–60°S | | | | | 213 | 73.9 | 12.6 | 13.5 | | | | |
| 60–70°S | | | | | 273 | 72.8 | 14.1 | 13.1 | | | | |

Note. The figures express the percentage ratio of water samples with the corresponding number of colonies to the total number of water samples examined.

The geographic patterns in the distribution of the microbial population (heterotrophs) in the Atlantic Ocean stand out especially clearly when comparing the percentage ratios of water samples which are poor and rich in heterotrophs at different latitudes (Table 1).

It is easily noted that, as one moves away from the equatorial-tropical regions, the percentage of water samples with a high concentration of heterotrophs decreases considerably, and the percentage of samples which are poor in these microorganisms rises sharply.

The Greenland (Fig. 4), and especially the Norwegian Seas (Fig. 5) are somewhat outstanding in their content of heterotrophic microorganisms, if they are compared with the subarctic regions (along 30° W) of the Atlantic Ocean. Of the 575 water samples collected in the Norwegian Sea, 65% contained a relatively large number of heterotrophs, and in this respect, it approximated the subtropical region of the North Atlantic. The southern and northern halves of the Norwegian Sea differed markedly: of the water samples from sections in the southern part, 27% gave growths of over 100 colonies, while in the northern part, only 2% of the water samples proved to be rich in heterotrophs. This peculiarity of the Norwegian Sea is undoubtedly explained by the considerable effect of the Gulf Stream on its hydrological character.

In the silts of the Atlantic Ocean and the Norwegian and Greenland Seas, tens, hundreds, and less frequently thousands of heterotrophic microorganisms were counted per g of undried silt.

A comparison of the results of investigation in the Atlantic, Indian, and Pacific Oceans (Table) enables one to conclude that there is a distinctly pronounced geographic zonality in the distribution, in the world ocean, of microbial forms utilizing easily assimilable organic matter in their vital activity. The significance of these heterotrophic microorganisms in oceanology is determined by the fact that their quantitative distribution makes it possible to judge the distribution in the seas and oceans of organic material which has not yet been transformed into humus and which is relatively easily available to the action of hydrolytic enzymes.

It now appears obvious that the regions of the higher latitudes of the world ocean—the arctic and antarctic, the subarctic and subantarctic – are regions where the density of the microbial population (heterotrophs) is lowest. Although these regions are the richest in plant life, which provides for the productivity of oceanic waters, the metabolic products of the organisms inhabiting these waters and their dead cells do not create such depots of nutrient material for heterotrophic microorganisms as occur in equatorial-tropical regions of the world ocean.

The concentration of microbial life (heterotrophs) in the examined portions of the equatorial and tropical zones of the Alantic, Indian, and Pacific Oceans is amazing high.† On a background of other geographical zones of the wold ocean, these zones are very outstanding in their richness in microbial life. It seems paradoxical that a high density of microbial population (heterotrophs) is observed in these geographic regions of the world ocean where the plant and animal population is poorest in comparison with the high latitudes.

Thus, whereas with respect to microorganisms, there is an increase in the content of heterotrophs in the water mass of the world ocean as one moves from the polar regions to the equator, with respect to other forms of life, the opposite picture is observed (Zenkevich, 1951) — an increase in concentration with distance from the equatorial-tropical zone.

The explanation for this paradoxical inconsistency must be sought in the fact that, in the tropical regions of the world ocean, the principal source determining the richness of the waters of these regions with regard to microbial population is slightly tranformed organic matter of allochthonic origin which has not yet undergone humification. It is also evident that,

†It must be taken into consideration that the method of cultivation on membrane ultrafilters gives a minimized ideal of the number of heterotrophs in waters where the content of these microorganisms is high (Kriss, 1959).

in the equatorial-tropical zone, the concentration of this organic material considerably exceeds that of analogous forms of autochthonic organic material in the water of high latitudes and of organic material entering the ocean from land there.

In the equatorial-tropical zone of the Pacific and Indian Oceans it must be assumed that the enrichment of the waters by organic material, which is easily available to the action of the hydrolytic enzymes of microorganisms, occurs in the Coral Sea and among the islands of the Australasian Archipelago. In the Atlantic Ocean, the increased concentration of slightly transformed organic material in the water of the tropical region is evidently caused by the run-off of such vast rivers as the Amazon and Orinoco, the Congo and the Niger, which gather water from extensive regions of equatorial America and Africa which are thickly populated by plant and animal life. A definite role as a source of allochthonic organic material must be ascribed to the numerous islands of the Caribbean Sea, into which offshoots of equatorial currents enter.

Kolbe's (1957) findings of the shells of fresh-water diatoms in silt deposits in the equatorial zone of the Atlantic Ocean are very interesting in this respect. In soil samples collected at a distance of 1000 kilometers from the African coast, the author found more than 1000 fresh-water diatoms (on one slide). Considering the great distance from land of the places where these diatoms were found, the great depth of the ocean, and the high concentration of diatom valves, it would be difficult to overestimate the influence of the runoff from equatorial Africa, which is picked up by trade wind currents, on the content of organic

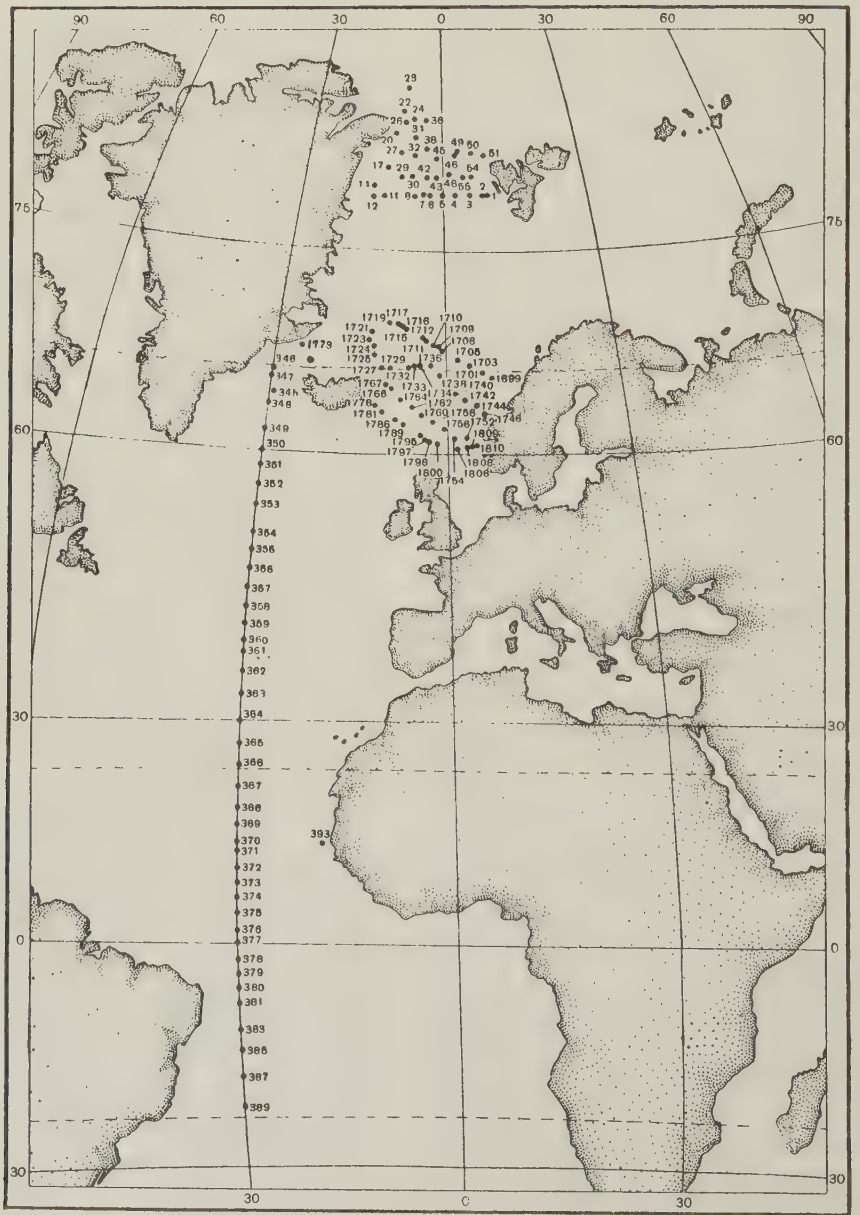


Fig. 1. A map of microbiological stations in the Atlantic Ocean and in the Norwegian and Greenland Seas. • — Site of station No. 1770.

and inorganic substances in the water mass of the equatorial-tropical zone of the Atlantic Ocean. Strong northeast winds, carrying such dense clouds of dust from the African continent that they sometimes limit visibility to 1-2 km or even 150 m in the region of the Atlantic Ocean adjacent to Africa between 15° N and 5° S, also serve as a means of transfer of terrigenous organic material to the waters of the northern and southern equatorial currents which start at the coast of Africa.

The equatorial-tropical waters enriched with easily assimilable forms of organic material for microorganisms are found in the subtropical and subarctic zones of the Atlantic Ocean where they are brought by currents. Clearly pronounced layers of this water were traced in the subtropical zone (23-40° N) at depths of: I) 10-25 m; II) 50-75, 75-100, 100-150, 200, and 300 m; III) 350-450, 450-500, and 600-750 m; IV) 400-600, 600-750, 800-950, and 850-1000 m; and layer V, which was observed at 35° N) 1000-2000, 1750-2250, and 1500 m. More northerly, between 40-66° N, continuations of the layers or separate "islets" of waters of equatorial-tropical origin were found at depths of from 10 to 1000 m. At two stations, increased numbers of heterotrophic microorganisms were detected in the 2500-3000 m layer (Figs. 2, 3, 6).

The predominance of waters with very low contents of heterotrophic microorganisms was characteristic for the section along 30° W in the entire water

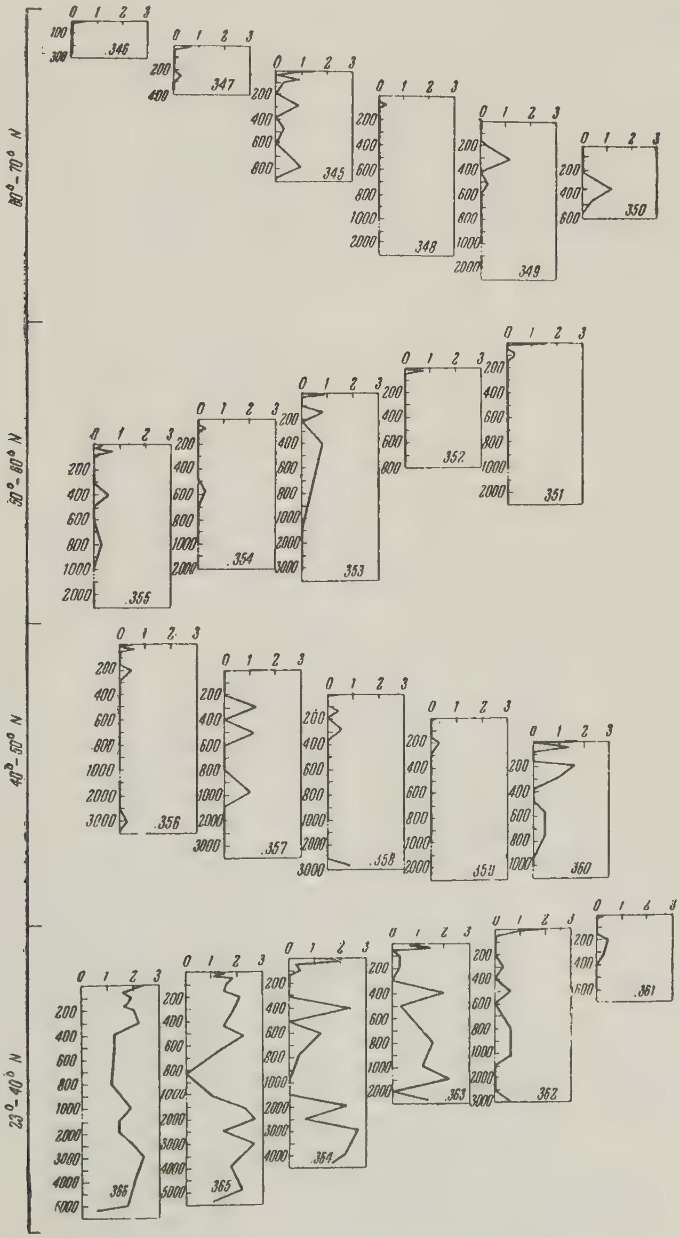


Fig. 2. Vertical distribution of heterotrophic microorganisms at microbiological stations in the Atlantic Ocean between 70° N and the Tropic of Cancer (the number of bacteria given is per 40 ml of water). Within each graph: Ordinates—depth (meters); abscissae—log of number of bacteria; station numbers as shown.

mass of the Atlantic Ocean from the Danish Straits to the Tropic of Cancer. The lack of slightly transformed nonhumified organic material is evidence of the significant effect on the hydrological structure of this part of the Atlantic Ocean of waters of arctic origin. These waters also penetrate to the tropical region. A layer with a small amount of heterotrophs was found between the Tropics of Cancer and Capricorn at depths of 1500, 750-1000, 850-1250, 700-950, 1000-1500, 1250-1750, 1500-2250, 2000, 1000-1750, 1000-1500, 1250-1750, and 1000-1500 m (Figs. 2, 3, 6).

It is important that the microbiological data coincide with Defant's hydrological data concerning the passage of waters of high-latitude origin through the Equator; however, these waters do not constitute as thick a layer in the tropical region of the Atlantic Ocean as on Defant's scheme (1957). So far, only the arctic origin of the given layer, which is impoverished with respect to forms of organic material which are easily assimilable by microorganisms, is uncertain. Taking into consideration that according to Defant's data, subantarctic waters cross the Equator in the 1000-2000 m layer and are found to 20°N, it can be assumed that, in the equatorial-tropical zone, the waters which are poor in heterotrophs are in some part mixed arctic-antarctic (subantarctic) waters.

Besides the high-latitude waters in the 750-2250 m layer, less pronounced layers with lower contents of

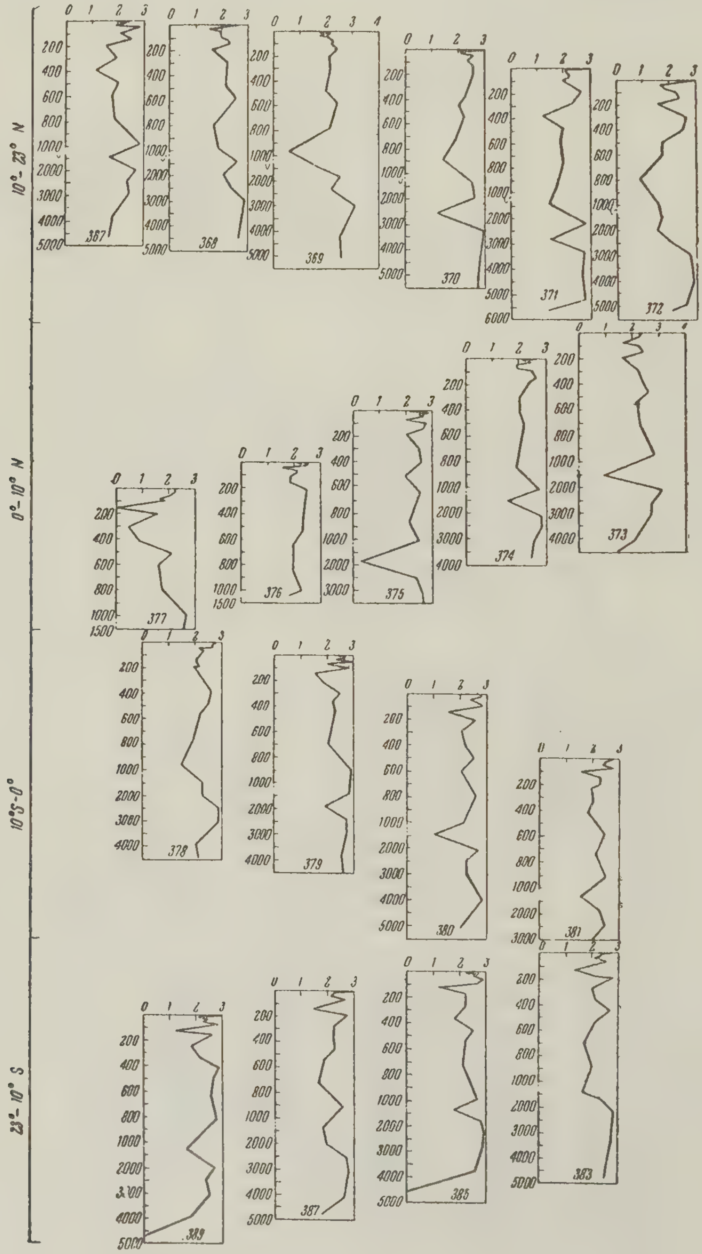


Fig. 3. Vertical distribution of heterotrophic microorganisms at microbiological stations in the Atlantic Ocean between the Tropics of Cancer and Capricorn (the number of bacteria is given per 40 ml of water). Designations within each graph same as in Fig. 2.

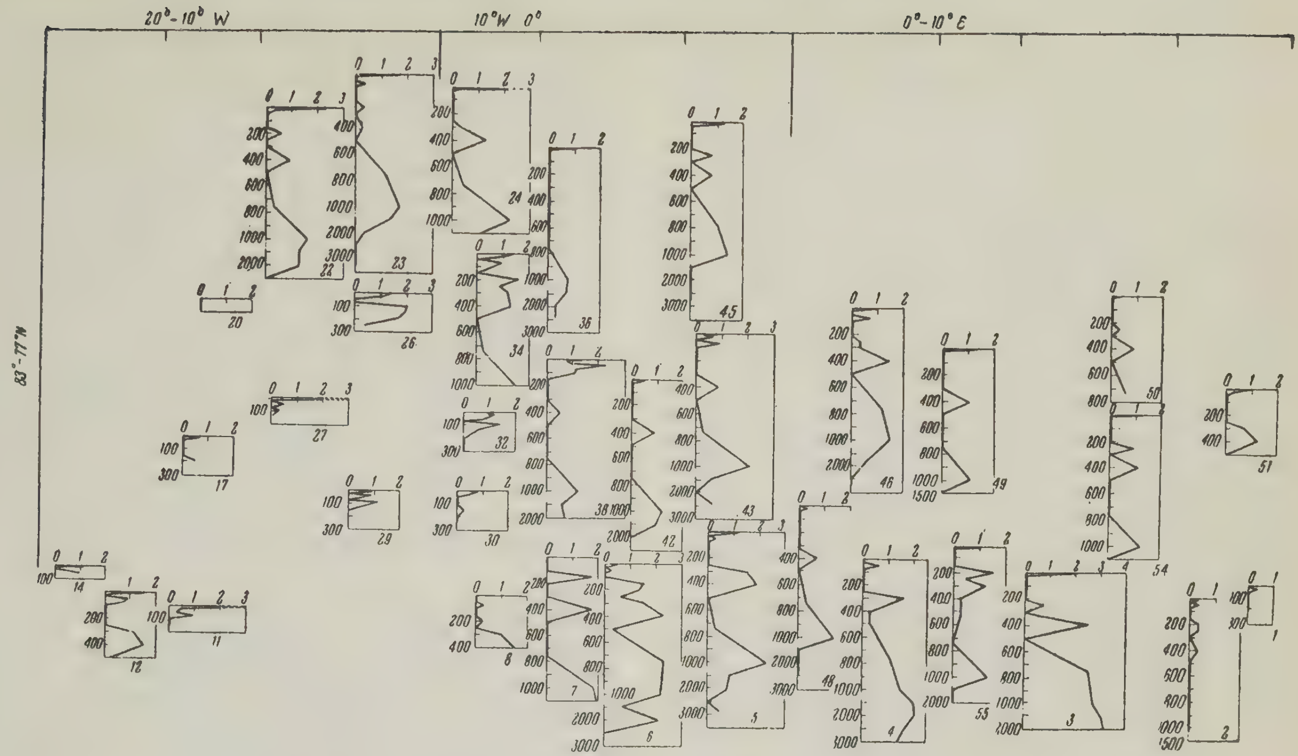


**Fig. 4.** Vertical distribution of heterotrophic microorganisms at microbiological stations in the Greenland Sea (the number of bacteria is given per 40 ml of water). Designations within each graph same as in Fig. 2.

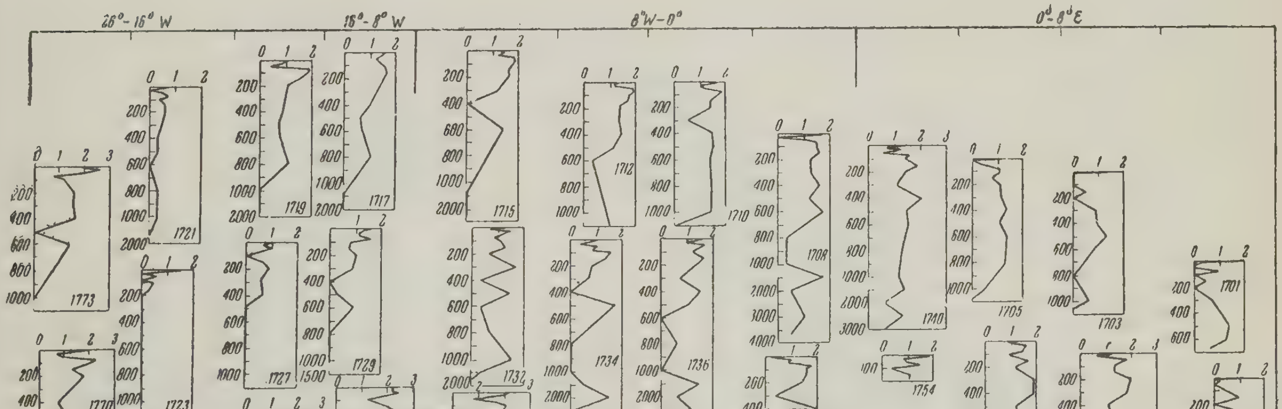


**Fig. 5.** Vertical distribution of heterotrophic microorganism at microbiological stations in the in the Norwegian Sea (the number of bacteria is given per 40 ml of water). Designations within each graph same as in Fig. 2.

heterotrophs are detected in the tropical region of the Atlantic Ocean at depths of 25, 50, 70 and 100-150 m. In the south tropical zone, these layers extend from 23° S nearly to the Equator. A relatively thick layer is formed by antarctic waters in the demersal region between the Tropic of Capricorn and 13-14° S.

As is the case in the subarctic region of the Atlantic Ocean, the principal mass of waters in the Greenland Sea (between 78 and 83° N) is represented by waters of arctic origin. However, at some depths, layers with a relatively high content of heterotrophs are encountered, which is evidence of the penetration into these regions of offshoots of the North Atlantic current carrying equatorial-tropical waters (Figs. 4, 6).

The layer enriched with heterotrophic microorganisms was encountered at a depth of 300-400 m at all stations except the shallow ones and station 36. In moving from 78 to 83° N, the concentration of heterotrophs in this layer decreased, but it was still considerably higher than in the water layers situated above and below it.

It is also interesting that in the Greenland Sea, rather thick layers of waters of equatorial-tropical origin were found at depths of 750-1000-2000 m. In some places, these waters extended to 3000 m and filled the demersal area: in others, arctic waters passed between these waters and the bottom.

At some stations, offshoots of the North Atlantic current were observed at depths of 150-200, 200-250 m, and in layers closer to the surface.

The hydrological structure of the Norwegian Sea is of a more complex character. The stratification taking place as a result of the passage of offshoots of the east Greenland and North Atlantic currents here is seen in Figs. 5, 6. Streams of these waters are shifted both in the surface layers and at great depths, but arctic waters were found at nearly all deep-water stations in the demersal area. In the layer to 1000 m the greatest mass of waters of equatorial-tropical origin was detected at stations between 0 and 8° W, which were topographically close to the channel of the main stream of the North Atlantic current in the Norwegian Sea.

In examining the microbiological data on the distribution of waters of various origins in the Norwegian Sea, it also becomes evident that equatorial-tropical waters exert their influence not only at depths to 1000 m. Rather thick layers of waters, characteristic in their increased content of organic material easily assimilable by microorganisms, were detected considerably deeper–in the 1000-2000 and 2000-3000 m layers.

The approximate correspondence of the boundaries of layers or "islets" of water of equatorial-tropical origin, which are rather consistently observed at depths of 300–400–600 and 750–1000–1500–2000 m in the Greenland and Norwegian (12-14° W) Seas and in the northern part of the Atlantic Ocean along 30° W, is quite striking. The circulation of equatorial-tropical waters is possibly of a rather stable character at these depths and encompasses considerable areas in

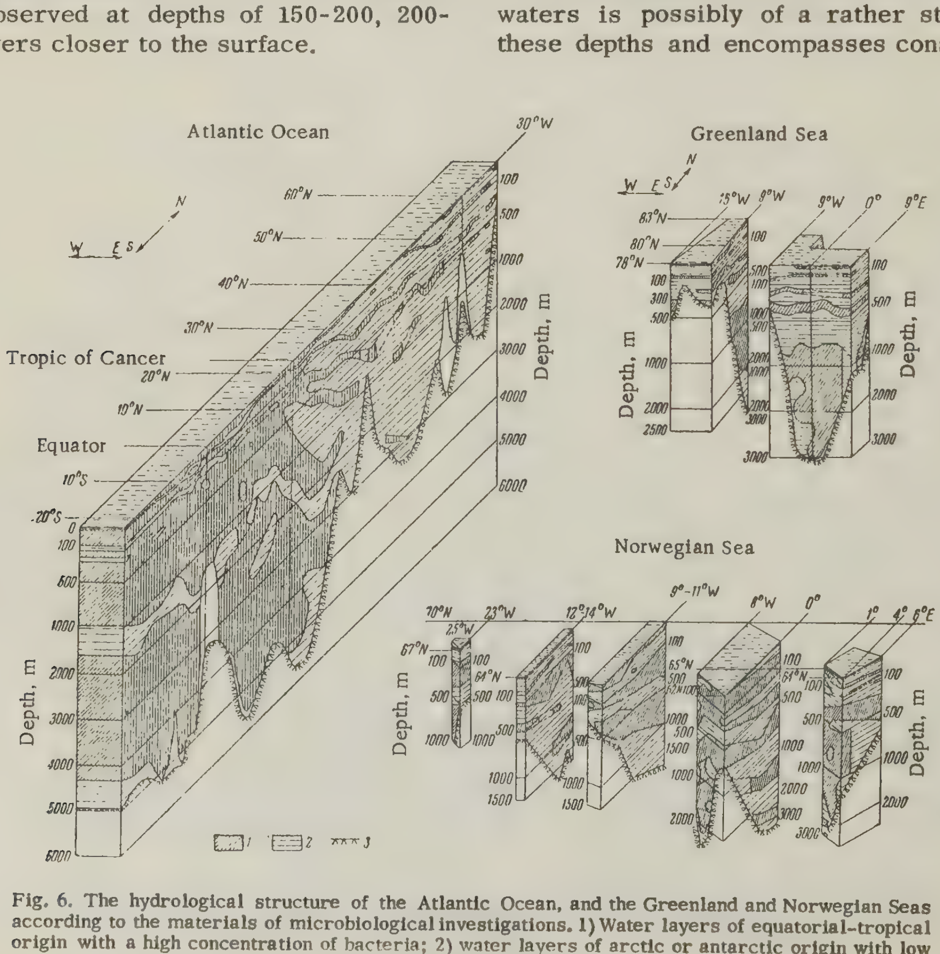


Fig. 6. The hydrological structure of the Atlantic Ocean, and the Greenland and Norwegian Seas according to the materials of microbiological investigations. 1) Water layers of equatorial-tropical origin with a high concentration of bacteria; 2) water layers of arctic or antarctic origin with low bacterial content; 3) sea floor.

the northern part of the Atlantic Ocean, in the Greenland Sea, and in the western regions of the Norwegian Sea.

Microbiological data do not permit one to subscribe to the widespread point of view concerning the absence of equatorial water masses in the Atlantic Ocean (Sverdrup, Johnson and Fleming, 1957) in distinction from the Indian and Pacific Oceans, where these water masses occupy the tropical regions. Judging by the great enrichment of the waters of the equatorial-tropical zone of the Atlantic Ocean by slightly transformed, still unhumified organic material, they acquire new distinguishing characteristics and, therefore, can hardly be regarded as merely transitional betwen the heterogeneous central water masses of the northern and southern parts of the Atlantic Ocean; rather, it should be considered probable that, as the result of considerable transformation, the waters of the equatorial Atlantic acquire specific characteristics which are in certain respects similar to the peculiarities of the equatorial water masses of the Pacific and Indian Oceans.

The currents carrying along the equatorial-tropical waters right up to the very highest latitudes are a factor in the distribution of terrigenous organic material throughout the water mass of the world ocean. Judging by the numbers of heterotrophic microorganisms in these waters, which mineralize organic material, the role of allochthonic organic material as a source of biogenic material ensuring the productivity of ocean waters must not be underestimated.

It must also be allowed that deep ocean currents carrying equatorial-tropical waters are by no means slow, as has been assumed to the present time. Otherwise, it would be difficult to explain the retention, in the deep layers of water in the temperate zone and at higher latitudes, of such concentrations of easily decomposed organic material which provides for such a considerable content of heterotrophic microorganisms in these layers.

## SUMMARY

1. The quantitative distribution of heterotrophic microorganisms in the water mass of the Atlantic Ocean between Greenland and the Tropic of Capricorn (along 30° W) and in the Norwegian and Greenland Seas was studied. The concentration of microorganisms assimilating slightly transformed, still unhumified organic material is very great in the tropical regions, while their content is low in subarctic and arctic regions.

2. Equatorial-tropical waters rich in heterotrophs were found in the subtropical and subarctic zones of the Atlantic Ocean and the Norwegian and Greenland Seas at various depths. These waters were detected not only in the layer to 1000 m, but considerably deeper – 2000-2500-3000 m, as well.

3. Judging by the correspondence of the boundaries of layers or "islets" of equatorial-tropical waters in the Atlantic, in the western part of the Norwegian Sea and in the Greenland Sea, the circulation of these waters at some depths is of a stable character and encompasses considerable areas.

4. The principal water mass of the Atlantic Ocean (along 30° W) from the Danish Straits to the Tropic of Cancer is comprised of waters of arctic origin which are very poor in heterotrophic microorganisms. These waters penetrate to the tropical region of the Atlantic Ocean and cross the Equator; however, they do not constitute as thick a layer in the equatorial-tropical zone as according to Defant's scheme.

5. Microbiological data suggest that the waters of the equatorial-tropical zone of the Atlantic Ocean are considerably enriched by slightly transformed, unhumified organic material. In this respect, they are similar to the equatorial water masses of the Indian and Pacific Oceans and can hardly be regarded as merely transitional between the central water masses of the North and South Atlantic.

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# MICROBIOLOGICAL PRODUCTION OF SULFUR FROM SULFIDE SEAM WATERS

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In sulfur and oil fields, particularly in the latter, seam waters containing as much as 1800 mg/liter sulfides and free hydrogen sulfide are brought to the surface and usually drain off into lakes and rivers or are pumped back again into the seam. This has given rise to the question of whether the sulfides in these waters can be oxidized to elementary sulfur, particularly since Ivanov's observations (1957) on the oxidation of hydrogen sulfide to elementary sulfur in Lake Sernoe in the Kuibyshev Region have shown that sulfur production there was due to the activity of Thiobacillus thioparus. According to his calculations the daily sulfur production of the whole lake was about 150 kg, the mean hydrogen sulfide content of the spring water being 86 mg/liter and the inflow 6 million liters per day.

This led Ashirov in the Giprovostokneft' Institute to raise the question of obtaining elementary sulfur from highly concentrated sulfide seam waters with a low oxidation-reduction potential.

A biological method of ridding deep waters of small quantities of hydrogen sulfide has already been devised by Pleshakov (1956).

It seemed to us also that the most efficient way of effecting this would be a biological method involving the use of a culture of T. thioparus.

Thus the present work entailed the development of a microbiological method of obtaining molecular sulfur in laboratory conditions and a test of this method in a plant operating with natural sulfide seam water.*

## SELECTION OF BACTERIAL CULTURES FOR EXPERIMENTS

For obtaining molecular sulfur from sulfides we tried first a pure culture of T. thioparus which we isolated from the mud of Lake Belovod', Vladimir Region (Kuznetsov and Sokolova, 1960). Several laboratory experiments were conducted with this culture. Since the seam water of the Kalinovka oil field, where we intended later to conduct the field experiments, contains up to 150 g of mineral salts per liter, the cultures of thiobacteria were tested for tolerance to NaCl by cultivation in Beijerinck's medium with different NaCl content. We found that our strain of T. thioparus could not tolerate a concentration of NaCl of more than 0.2%. Hence before we began our experiments with seam water we had to isolate a new culture of bacteria. After several experiments we managed to isolate a pure, very active culture of thiobacteria from the seam water in well No. 240 in Kalinovka. The culture was isolated in Beijerinck's medium prepared with 1:1 diluted seam water.

This T. thioparus also grew well in Beijerinck's medium with the addition of NaCl in amounts of 10–150 g/liter. The best growth was observed at 50–100 g/liter. When the NaCl content was less than 1% there was no growth of this strain of thiobacterium.

## METHOD

A count of T. thioparus was made by the dilution method with liquid Beijerinck's medium. The growth of the bacteria was determined from the appearance of a sulfur film and from a microscopical examination of the specimens.

A chemical analysis for sulfates was carried out daily by direct titration with alizarin S (Zavarov, 1957). The quantity of molecular sulfur was determined by the combustion technique devised by the State Institute of Mined Chemical Raw Materials. Sulfides and hyposulfite were determined by iodometric titration. The pH was measured by a LP-6 potentiometer with a glass electrode and the oxidation-reduction potential with a smooth platinum electrode.

As our laboratory investigations showed (Kuznetsov and Sokolova, 1960) T. thioparus is not a strict aerobe. In pure culture it remains viable at a very low oxidation-reduction potential ($rH_2$ = 5–8) and hyposulfite oxidation is most intense in a medium with low values of the potential, in the range $rH_2$ = 12–18.

This organism was found at the same values of potential in Shor-Su by Ivanov (1960), in oil deposits in Grozny region by Al'tovskii, Kuznetsova, and Shvets (1958), and by us in the Yazovo sulfur deposits. Thus the first problem was to devise continuous-flow experiments at $rH_2$ = 16–18.

The laboratory experiments on sulfur production were carried out in an apparatus consisting of two communicating tall 0.5-liter cylinders through which passed a steady flow of nutrient medium containing hyposulfite or calcium sulfide. The first cylinder was inoculated with a culture to give 20 million cells per liter. Air was blown through from below and passed

*We received great assistance in the field work from M. V. Makarenko, the Head Geologist of the Oil Industry Board "Kinel'neft'," P. V. Tokin, and the workers under foreman N. E. Shcherbakov in section I of the first oil field in Kalinovka, and we regard it as a pleasant duty to express our thanks to them.

Table 1. Obtention of Molecular Sulfur from Calcium Sulfide
(aeration rate: 1 volume of air per 1 volume of medium per hr, data in mg S/liter)

| Time, days | Experimental | | | | | Control | | | |
|---|---|---|---|---|---|---|---|---|---|
| | S/CaS | $S/SO_4''$ | S molec | pH | $rH_2$ | S/CaS | $S/SO_4''$ | S molec | pH |
| 0 | 219 | 131.2 | 0 | 8.85 | 12.5 | 224 | 108.8 | 0 | 8.80 |
| 1 | 16 | 131.2 | 192 | 9.05 | 18.7 | — | — | — | — |
| 4 | 0 | 297.6 | 32 | 7.50 | 19.8 | 198 | 112.2 | 0 | 9.10 |

through the whole column in the form of tiny bubbles. Here the oxidation of sulfur compounds took place with the formation of molecular colloidal sulfur. In the second cylinder this sulfur was precipitated by the periodic addition of a coagulant consisting of $Al_2(SO_4)_3$ or by strong alkalization of the medium with caustic potash to pH 12.5. The sulfur content of the precipitate was analyzed. To find out the most suitable conditions of operation of the apparatus we first carried out experiments with a hyposulfite-containing medium in which the thiobacteria grew well.

## RESULTS OF LABORATORY EXPERIMENTS

The results of the analyses are given in Fig. 1.

Hyposulfite was oxidized at a high rate at low potentials. As Fig. 1 reveals, with a weak flow of air at a rate of one volume of air per volume of medium per hour the potential fell sharply from the very start of the experiment. The oxidation of hyposulfite with the production of sulfur proceeded as the $rH_2$ fell from 24 to 14 and lower and soon terminated. When the aeration was increased to 10 volumes of air per hour the bacteria developed slowly, there was no reduction in the $rH_2$, and it was not until the fourth day, when the $rH_2$ fell below 22, that an appreciable oxidation of hyposulfite began and molecular sulfur was produced.

Finally, with strong aeration at a rate of 60 volumes per hour the potential remained at 22 for a week and it was not until the 12th day, when the potential fell to 16, that the hyposulfite began to undergo rapid oxidation.

The following series of experiments on the oxidation of sulfides was carried out. As experiments with T. thioparus indicated, there was practically no oxidation of sodium sulfide. In this case, however, there was still a weak growth of bacteria at the expense of the oxidation of molecular sulfur, which appeared in the medium when the sodium sulfide was chemically oxidized by the oxygen of the air.

In contrast to this, the development of thiobacteria with the production of elementary sulfur took place when the sodium sulfide was replaced by calcium sulfide. The results of the experiment on the oxidation of calcium sulfide are given in Table 1, where we see that practically all the sulfide was oxidized to sulfur within 24 hr and some was even oxidized to sulfate, whereas in the sterile control subject to the same rate of aeration there was no formation of sulfur.

A count of the thiobacteria showed that the experimental cylinder contained up to $10^8$ cells/ml.

Thus the laboratory experiments showed that when T. thioparus was grown in a weakly aerated medium containing calcium sulfide the sulfides were rapidly oxidized to form colloidal molecular sulfur, which was precipitated by coagulation and accumulated in the settling cylinder. Analyses showed that 38.4% of the dry weight of this deposit was sulfur.

## EXPERIMENTS WITH SULFIDE SEAM WATER

The experiments on obtaining sulfur from oil-seam sulfide waters were carried out in the Kalinovka oil field, Kuibyshev Region. The apparatus used is shown schematically in Fig. 2. It consists of three connected 40-liter cylinders. The first cylinder was fed with fresh seam water containing 1.2 g of sulfides per liter.

Dispersed air was passed through the first two cylinders from below by means of a perforated spiral tube. The third cylinder was used for coagulation and

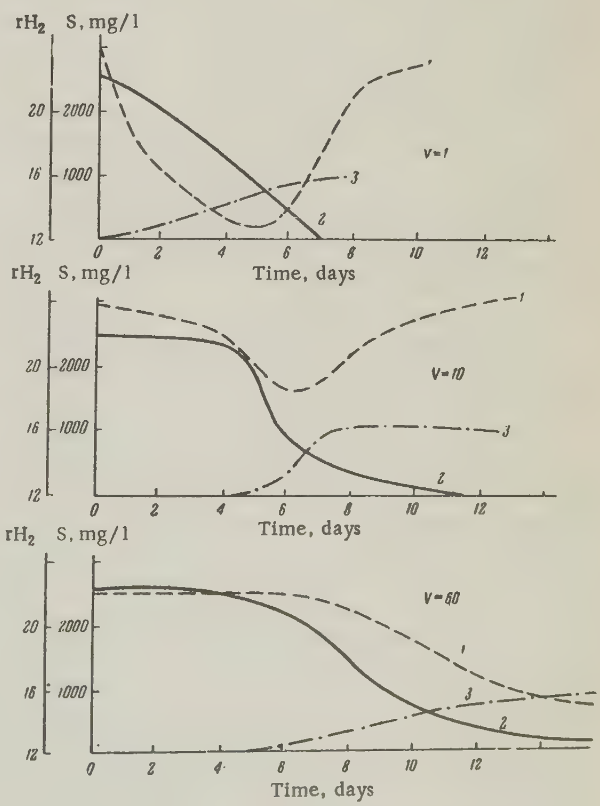


Fig. 1. Hyposulfite oxidation by Thiobacillus thioparus in relation to aeration rate. 1) rH ; 2) hyposulfite content of medium; 3) production of molecular sulfur; 4) aeration rate in volumes of air per volume of medium per hr.

Table 2. Chemical Analyses of Sulfur Compounds and Variation of pH and $rH_2$ of Seam Water During Oxidation in a Field Plant

| Duration of expt, days | Cylinder I | | | | | Cylinder II | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | pH | $rH_2$ | mg/liter | | | pH | $rH_2$ | mg/liter | | |
| | | | S'' | S/$SO_4''$ | S molec | | | S'' | S/$SO_4''$ | S molec |
| 0 | 7.40 | 8.40 | 1143 | 1493 | 0 | 7.50 | 9.45 | 1135 | 1488 | 0 |
| 1 | 8.50 | 14.93 | 192 | 1728 | 492 | 8.55 | 14.03 | 185.6 | 1748 | 476 |
| Flow started in plant | | | | | | | | | | |
| 2 | 8.65 | 16.96 | 102 | 1760 | — | 8.10 | 22.65 | Trace | 1881.6 | — |
| 3 | 8.30 | 13.20 | 99.2 | 1792 | — | 8.00 | 24.62 | " | 1920 | — |
| 4 | 8.45 | 11.35 | 128.6 | 1827 | 835 | 8.10 | 25.92 | 0 | 1752 | 710 |

precipitation of the sulfur. The sulfur, together with the hydrates formed by alkalization, settled out and was removed through the stopcock at the bottom. The conditions of operation of the apparatus were decided on by reference to the results of the laboratory experiments. Air was delivered at a rate of 2–3 volumes per volume of water per hr. The seam water flowed through at a rate of 1 volume per 24 hr so that in passage through the two cylinders the sulfides were completely oxidized. Each of the two cylinders was inoculated daily with 1.5 liter of fresh active culture of T. thioparus isolated from seam water from the same well feeding water to the apparatus.

The bacterial content of the water itself was not very high, 1 cell/ml. Before we succeeded in isolating a pure bacterial culture the apparatus was operated without bacteria and with no flow of water. During the first week there was no formation of sulfur at all and it was not until after two weeks that a yellowish film of sulfur appeared on the walls of the cylinders. The bacterial content by this time had risen to $10^3$ cells/ml. These results served as a control for the experiment and were convincing evidence that the formation of sulfur in the experiment took place at low pH due to the activity of T. thioparus and not as a result of chemical oxidation of the sulfides when the air was passed through.

The data of the analyses are collected in Table 2.

The experiments showed that after the introduction of the bacteria into the seam water a very rapid oxidation of sulfides began and the medium became very turbid owing to the colloidal sulfur produced. Within 24 hr the sulfides had been oxidized to sulfur and part of the sulfur to sulfates. When the flow was started in the apparatus at a rate of 1 volume of water per day the bulk of the sulfides were oxidized in the first cylinder, and water flowed through the second cylinder with a sulfide content of 100–200 mg/liter. This sulfide was oxidized in the second cylinder and $H_2S$-free water passed into the settling cylinder. In this case however, some of the sulfur was over oxidized to sulfates, particularly in the second cylinder. When the flow rate was increased sulfides arrived in the settling cylinder. It would seem that if the chain of connected vessels was made longer it would be possible to increase considerably the rate of flow of seam water through the plant as well as to prevent excessive overoxidation of the sulfides to sulfates.

Analyses performed daily over several weeks revealed that the process took place under the same oxidation-reduction conditions as in the laboratory experiments. In the first cylinder the potential remained in the range 11–17 and in the second it was higher, 14–24. The sulfides were oxidized to sulfur at a rate of approximately 700–800 mg/liter per day, but about one-third of the sulfur was converted to sulfates, so that the final production was 0.5 g S/liter per day.

The rate of sulfide oxidation can be much higher. Experiments have shown that a young culture of T. thioparus can oxidize as much as 1350 mg of hyposulfite sulfur per liter per day.

Experiments showed that a fresh culture of bacteria had to be introduced into the cylinders every day since at a high rate of water flow the culture could be washed out, especially from the first cylinder. Under the above conditions of operation of the apparatus the bacterial content of the first cylinder reached 10 million cells/ml and in the second cylinder 100 million cells/ml. Since the culture added to the cylinders also contained about 10 million cells/ml it would probably be possible to simplify the method of inoculation and transfer 1–liters of the liquid from the second cylinder into the first.

Sulfur constituted 6.5% of the dry weight of the formed deposit. This small percentage was attributed to the precipitation of a large quantity of salts from the seam water on alkalization.

Thus the conducted experiments showed that when T. thioparus grows in natural seam water containing

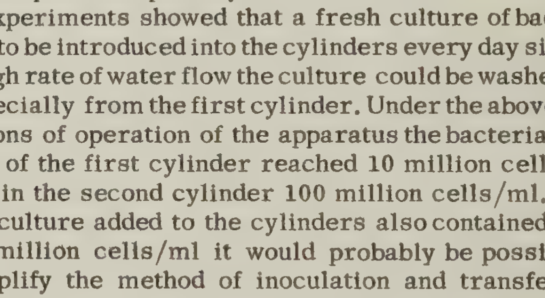


Fig. 2. Schematic of pilot-plant apparatus for obtaining sulfur from sulfide seam water.

1.2 g $H_2S$/l the oxidation of sulfides takes place with the production of 0.5 g $H_2S$/l on the average, and we must assume that the rate of sulfur production could be considerably increased by altering the design of the apparatus.

As Ashirov (1959) pointed out, the extraction of oil entails the extraction of huge quantities of seam water. As time goes on the quantity of water extracted with the oil increases considerably, reaching 99% of the total volume. Hence the further exploitation of such deposits for oil becomes uneconomic.

However, seam water constitutes a valuable raw material for obtaining several chemical products—I, Br, Mg, Cl, S, and others. In the forthcoming years more than 100,000 tons of seam water, containing up to 20 tons of sulfide sulfur per day, will be extracted daily from the oil fields of the Kuibyshev Region alone. The development of the technology of sulfur production from sulfide waters on an industrial scale could greatly benefit our sulfur industry. It would also promote the maximum utilization of industrial sites and the economic exploitation of several old flooded oil fields.

This method could also be employed for the purification of industrial sulfide wastes.

## SUMMARY

1. A pure Thiobacillus thioparus culture was isolated from seam waters of the Kalinovka oil field. This culture could grow in a medium containing 10 to 150 g NaCl/l and was subsequently used for the production of sulfur from sulfide seam waters.

2. A technique was developed for the production of elementary sulfur by using a pure culture of T. thioparus in artificial media containing calcium sulfide.

3. A field test of this biogenic technique of obtaining sulfur from sulfide seam water with a sulfide content of 1.2 g/liter showed that free sulfur could be produced at a rate of 0.5 g/liter of seam water per day.

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# MEDIUM FOR AMYLASE AND PROTEINASE PRODUCTION BY A SUBMERGED CULTURE OF BACILLUS SUBTILIS

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Bacillus subtilis is used for obtaining amylase and proteinase preparations. We began our study of amylase and proteinase production by a submerged culture of B. subtilis by selecting the best medium for the synthesis of these enzymes. For this purpose we compared some of the media recommended in the literature for the cultivation of these bacteria.

## METHOD

In our work we used the Japanese strain of B. subtilis, described earlier (Feniksova et al., 1960). The bacteria were cultured at 37° C in 100 ml of medium in 250-ml flasks on a shaker.

We tried the following media:

1. Fukumoto's medium (1957): starch—5%, sodium citrate—0.5%, $(NH_4)_2PO_4$—1%, NaCl—0.2%, $MgSO_4 \cdot 7H_2O$—0.02%, KCl—0.01%, $CaCl_2$—0.01%; pH—7.3.

2. Soya medium (Nomura, 1955): starch—8%, sodium citrate—0.04%, $(NH_4)_2HPO_4$—0.15 M, KCl—0.02 M, $MgSO_4 \cdot 7H_2O$—0.002 M, CaCl—0.001 M, ethyl alcohol—1%, soybean extract*—5%; pH—7.2.

3. Medium "g" (Nomura, 1955a): starch—0.2%, glucose—2%, succinic acid—0.5%, $(NH_4)_2HPO_4$—0.025 M, KCl—0.002 M, $MgSO_4 \cdot 7H_2O$—0.002 M, $CaCl_2$—0.001 M, peptone—0.5%, soybean extract—0.5%; pH—7.

4. GB medium (Nomura, 1955a): glucose—0.5%, peptone—1%, meat bouillon—0.5%, NaCl—0.2%; pH—7.0.

5. SB medium (modified GB medium): starch—0.4%, peptone—0.9%, meat bouillon—0.4%, NaCl—0.2%, $CaCl_2$—0.01 M; pH—7.2.

6. LBY medium (Nomura, 1955b): peptone—1%, meat bouillon—0.5%, yeast extract—0.2%, NaCl—0.2%, lactose—1%.

7. 20% bran extract. For preparation of this medium 200 g of wheat bran was extracted with 1 liter of tap water for 1 hr in a boiling water bath. The mixture was then strained through cloth.

The amount of amylase contained in the culture fluid after the growth of the bacteria was determined by the method of Klimovskii and Rodzevich (1949) from the hydrolysis of starch to dextrins not staining with iodine. The amylolytic power was expressed in AP units. The proteolytic activity (in PA units) was determined by Ivanov's nephelometric method (1960) from the quantity of gelatin left after proteolysis. The tables and figures give the mean data obtained in two to three experiments of two repetitions.

## EXPERIMENTAL

A comparative study of the rate of amylase and proteinase formation by a submerged culture of B. subtilis was made with several media, mainly taken from the works of Japanese authors (Nomura, 1955a, b; Fukumoto, 1957a, b). These media as described above provided a good growth of bacteria but the amylase and proteinase production was different for the different media in which the bacteria grew. The two enzymes were formed best in soya medium (378 AP units and 243 PA units per 100 ml of culture fluid after two days of bacterial growth). A fairly high enzymatic activity was found when the bacteria were grown in lactose-bouillon-yeast medium (LBY medium) (314 AP units and 268 PA units) and in medium "g" (141 AP units and 229 PA units). The 20% yeast extract gave a relatively good yield of enzymes only after three days (230 AP units and 241 PA units). On the second day the enzyme production in this medium was low (67 AP units and 177 PA units).

On a more careful examination of the results given in Table 1 we note certain features in the production of enzymes by B. subtilis. Amylase is synthesized in considerable quantities not only in response to the presence of the specific substrate (starch) in the medium but also in response to other sources. For instance in LBY medium the main carbon source is lactose and in this medium the bacteria gave almost as much amylase as in the starch-containing soya medium. Yet in starch-bouillon medium (SB) and in Fukumoto's medium, despite the presence of starch in them, there was very little amylase or none at all. Considerable variations were also found in the amount of proteinase formed although some of the media contained the same nitrogen source, peptone.

We selected two media for a more thorough study, the soya medium, which was best for the production of the enzymes in question, and the bran extract. The latter medium was attractive in view of its availability and simplicity of preparation, an important feature for its use in industry.

A study of the dynamics of amylase and proteinase production in these media showed that the bulk of the amylase (82%) and proteinase (77%) in soya medium was formed by the 48th hr of growth of the culture

*The soybean extract for 1 liter of medium was prepared in the following way: 50 g of soybeans was extracted into 250 ml of 0.1% Na... by boiling for one hr.

Table 1. Production of Amylase and Proteinase by a Submerged Culture of B. subtilis in Various Media

| No. of medium | Medium | Enzymatic activity per 100 ml of culture fluid | | | |
|---|---|---|---|---|---|
| | | amylase (AP units) | | proteinase (PA units) | |
| | | two days of growth | three days of growth | two days of growth | three days of growth |
| 1 | Fukumoto | 72 | 65 | 65 | 70 |
| 2 | Soya | 378 | 403 | 243 | 285 |
| 3 | Medium "g" | 141 | 212 | 229 | 229 |
| 4 | GB | traces | traces | 48 | 41 |
| 5 | SB | " | " | 28 | 41 |
| 6 | LBY | 314 | 196 | 268 | 218 |
| 7 | 20% bran extract | 67 | 230 | 177 | 241 |

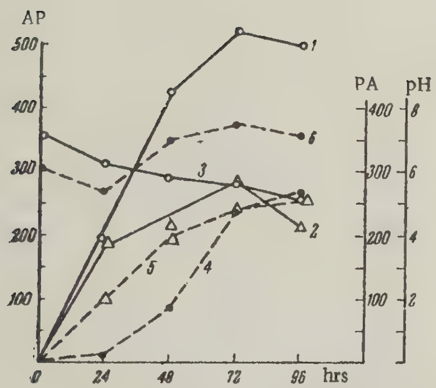


Fig. 1. Dynamics of amylase and proteinase production by a B. subtilis culture in soya medium and in bran extract. 1) Amylase in soya medium; 2) proteinase in soya medium; 3) pH; 4) amylase in bran extract; 5) proteinase in bran extract; 6) pH.

There was some increase up to the 72nd hr, after which the amount of enzymes decreased a little. In the bran extract amylase was synthesized very slowly and even by the fourth day of bacterial growth the amount was considerably lower than the yield in the soya medium. The synthesis of proteinase was practically the same in both media (Fig. 1).

We made attempts to increase the amylase production of a two-day culture of B. subtilis in bran extract by adding to the medium various constituents of the soya medium. However, these attempts met with no success.

The soya medium, which gave a very high yield of amylase and proteinase, had a fairly complex composition. Many of its ingredients were present in excess so that they were not completely used up by the bacteria and constituted ballast in the subsequent purification of the enzymes from this medium. Hence we decided to simplify the composition of the medium but in such a way that enzyme synthesis was reduced as little as possible. The results of these experiments are given in Table 2. A fourfold reduction in the starch content of soya medium (from 8 to 2%) was useful, since the amylase production was reduced by only 20% and that of proteinase by 33%. The absence of sodium citrate from the medium did not reduce the synthesis of the enzymes; therefore in later experiments this substance was left out. The preparation of soya medium with phosphate buffer instead of water, as recommended by certain authors (Fukumoto et al., 1957b), had no effect, and hence in all our experiments this medium was prepared from distilled water. We found that halving the quantity of mineral nitrogen in the medium led to a marked reduction in the synthesis of amylase and proteinase. The enzyme yield was greatly affected by the exclusion of the soya extract from the medium, reducing amylase production by a factor of 4.5 and halving proteinase production.

Figure 2 reveals, however, that a fourfold reduction of the soya extract with 8% starch present had practically no effect on the synthesis of either enzyme. On the other hand, amylase production fell very considerably when the soya extract was reduced by the same amount with 2% starch present. We may conclude that these two constituents of the medium largely compensate one another. At the same time a quite distinct tendency observed here was that a large reduction in the amount of soya extract in the medium of low starch content had a greater effect on the amylase synthesis (diminution of activity by four-fifths) than the proteinase synthesis (diminution by only one-third).

We know from the literature (Feniksova and Dvadtsatova, 1959) that an extract of malt sprouts stimulates amylase production by molds. In concluding this work we conducted experiments in which a 10% extract of malt sprouts was added to the soya medium, replacing the soya extract by the malt extract. We found that the soya extract had a more stimulating effect on bacterial amylase and proteinase production than the extract of malt sprouts (Fig. 3).

In view of all the data cited above, we settled on the soya medium with 2% starch and a full complement of soya extract as the most suitable of all the tested modifications of the medium. In addition, the sodium citrate was completely excluded from the medium. This medium insured an adequate production of amylase and proteinase (300–340 AP units and 200 PA units per 100 ml of culture fluid). The wet enzyme preparation obtained in this case contained 1300 AP units and 3200 PA units per 1 g of dry preparation. The yield of enzyme preparation was 1.2 g/l of culture fluid.

Table 2. Effect of Exclusion of Different Constituents of Soya Medium on Synthesis of Amylase and Proteinase by a 48-hr Culture of B. subtilis

| Medium | pH | | Enzyme yield per 100 ml of culture fluid | |
|---|---|---|---|---|
| | initial | final | amylase (AP units) | proteinase (PA units) |
| Unmodified soya medium with 8% starch | 7.2 | 6.1 | 453 | 285 |
| The same with 6% starch | 7.2 | 6.2 | 376 | 232 |
| The same with 4% starch | 7.2 | 6.2 | 392 | 197 |
| The same with 2% starch | 7.2 | 6.5 | 364 | 191 |
| Soya medium without sodium citrate | 7.2 | 5.8 | 467 | 261 |
| Soya medium prepared with phosphate buffer 1/30 M, pH 7.2 | 7.2 | 6.4 | 380 | 249 |
| Soya medium with half quantity of $(NH_4)_2HPO_4$ | 7.2 | 5.8 | 180 | 197 |
| Soya medium without soybean extract | 7.2 | 6.3 | 98 | 139 |

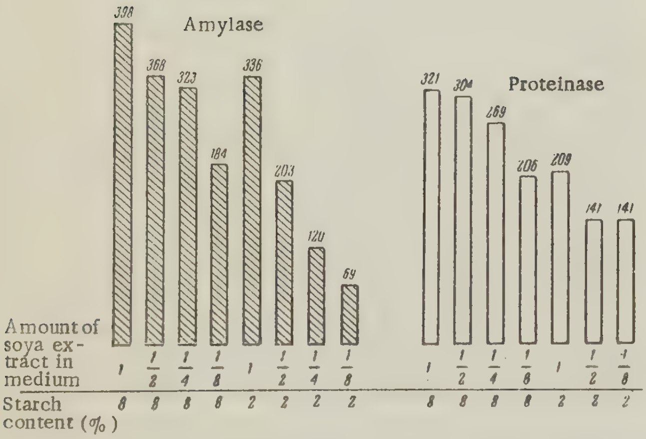


Fig. 2. Effect of reduction of amount of soybean extract and starch on amylase and proteinase production by a submerged culture of B. subtilis.

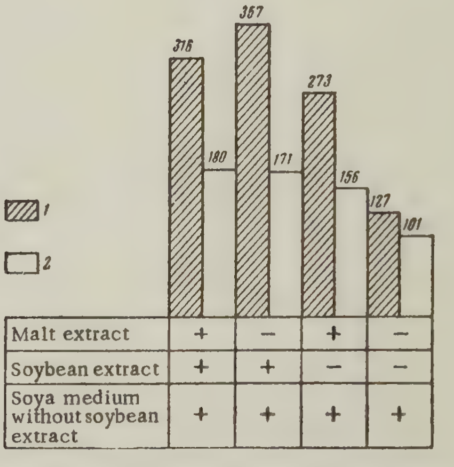


Fig. 3. Effect of soybean extract on synthesis of amylase and proteinase by a submerged culture of B. subtilis. 1) amylase (in AP units per 100 ml of culture fluid); 2) proteinase (in PA units per 100 ml of culture fluid).

## SUMMARY


1. The best medium for the synthesis of amylase and proteinase by a submerged Bacillus subtilis culture is the soybean medium suggested by Nomura.

2. The enzymes are present in greatest quantities on the third day of bacterial growth in this medium but 82% of the amylase and 77% of the proteinase are synthesized within two days.

3. The starch content of the soya medium can be reduced to a quarter. This considerably reduces the enzyme synthesis (amylase by 20% and proteinase by 33%) but it frees the medium from ballast substances and makes it more suitable for subsequent isolation and purification of the enzyme preparations. The complete exclusion of sodium citrate from the medium has no effect on the production of amylase or proteinase.

This modified soya medium insures a fairly high yield of amylase (300–340 AP units per 100 ml of culture fluid) and proteinase (200 PA units).

4. Synthesis of bacterial amylase and proteinase is greatly stimulated by an alkaline soybean extract. An aqueous extract of malt sprouts has a somewhat less stimulating effect.


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# EVOLUTION OF HEAT BY CULTURES OF THE MOLD ASPERGILLUS ORYZAE IN ENZYME PRODUCTION

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During their growth mold fungi liberate a considerable amount of physiological heat into the surrounding medium. This fact assumes particular importance when the fungi are cultivated for the industrial production of amylolytic and proteolytic enzymes.

The rise in temperature in the culture vessel results in overheating of the industrial culture, thus inhibiting the vegetative growth of the fungus and greatly reducing its ability to produce enzymes. The timely removal of the excess physiological heat from the culture vessels is thus extremely important in industry. Proper tackling of this problem requires a knowledge of the amount of heat liberated by the fungus during its growth.

Terroine and Wurmser (1922), Terroine and Bonnet (1926), Tauson (1950), and other authors (e.g., Famiya, 1932, 1933) have amassed a considerable amount of experimental data on the bioenergetics of molds. From these they have made important theoretical generalizations and determined several coefficients characterizing the quantitative aspect of the bioenergetic process. Unfortunately these data cannot be used directly in the enzyme industry, since the conditions of cultivation of the fungus on the industrial scale are substantially different from those which prevailed in the laboratory experiments of these authors. The fundamental principles established and the general bioenergetic principles still retain their value in application to industrial conditions and we have used them as methodological premises in conducting these investigations.

It is recognized that for such heterotrophic organisms as molds, the system "organism—nutrient medium" can be regarded as a closed system not only as regards metabolism but also as regards energy exchange. The energy required for the development and vital activity of the fungus can thus only be provided by that part of the potential energy of the nutrient medium released from the substances consumed or their conversion products. This energy is used up in the synthesis of substances forming the body structure of the organism itself as well as on "maintenance" (Terroine) or "basal metabolism" (Tauson), i.e., the accomplishment of all the other vital functions of the organism not involved in the production of biomass (on the internal work of the cells and the external, mainly mechanical, work of the organism during its development). Some of the energy released as a result of catabolic reactions is not used by the organism and is liberated as heat into the surrounding medium. The relative energies expended on the structure of the organism, its basal metabolism, and heat evolution may vary considerably with the species of fungus, its conditions of cultivation, and the composition of the medium used. The energy utilization coefficients for synthesizing activity are appreciably reduced when proteins and fats are used as a source of carbohydrate nutrition. For instance, for carbohydrates these coefficients are 0.58–0.60, for fatty acids, 0.43–0.47, and for proteins and amino acids, 0.36–0.39. As these coefficients diminish the fraction of energy released as heat increases accordingly.

For the industrial production of enzyme preparations, specially selected strains of the mold Aspergillus oryzae are used. They are cultured on a solid wheat bran nutrient medium spread on trays with a meshwork bottom. The duration of cultivation is 24 hr (Kalashnikov, Lifshits, Levintan, and Trainina, 1954). The culture vessels used in our enzyme factories (Khar'kov, L'vov, Rostov-on-Don) each receive a load of nutrient equivalent to 600–700 kg of air-dried bran for conversion to mold bran. The relatively low cubic capacity of these vessels necessitates a load nearing capacity and a high rate of ventilation to remove the excess heat.

The literature contains practically no information on the evolution of heat by the fungus in the conditions prevailing in the enzyme industry. We know of only one work (Underkofler, Sevenson, Goering, and Christensen, 1947) which contains references to the quantity of air which should be supplied to the culture vessel to remove excess heat and insure the normal development of the fungus. According to the data of these authors the quantity found empirically was very high. One kg of mold bran required an average of 54 $m^3$ of air per hr during the whole cycle of growth (36 hr in these authors' experiments) and the quantity rose to 80 $m^3$ per hr at the peak period.

A more reliable and more soundly based rate of ventilation of the culture vessels can be determined from experimentally obtained data on the heat evolution of the fungus.

In our investigations we undertook a study of the question of heat evolution by the fungus under conditions corresponding to those in the enzyme industry. It was important to investigate not only the total amount of heat liberated by the fungus during its whole cycle of growth but also the dynamics of the heat evolution,

Total Amount of Heat Liberated by A. oryzae During Whole Cycle of Growth (24 Hr in the Laboratory) (determined by calorimetric method and expressed in terms of 1 kg of dry bran)

| Expt. No. | Conditions of cultivation | No. of strain | Yield of prep., % of abs. dry substance (p) | Calorific value of abs. dry bran, kcal/kg ($Q_1$) | Calorific value of abs. dry prep., kcal/kg ($Q_2$) | Energy store in enzyme prep. from 1 kg bran, kcal ($Q_2 \cdot p/100$) | Heat evolution (kcal/kg bran) ($Q = Q_1 - \frac{Q_2 \cdot p}{100}$) | Catabolized matter per kg dry bran, in g (a = 1000 - 10 p) | Heat liberated per kg catabolized matter, kcal/kg (Q : a) | Activity of preparation ("AP" units) |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
| 1 | Indus- | 476-I | 89 66 | 4603 | 4713 | 4226 | 377 | 103 | 3.64 | 118 |
| 2 | trial | | 89.25 | 4602 | 4636 | 4182 | 420 | 103 | 3.90 | 118 |
| 3 | | | 79.36 | 4492 | 4663 | 3701 | 962 | 206 | 4.66 | 134 |
| 4 | | | 77.40 | 4627 | 4704 | 3641 | 986 | 226 | 4.36 | 130 |
| 5 | Labora- | 476-I | 84.20 | 4617 | 4738 | 3989 | 628 | 153 | 3.90 | 192 |
| 6 | tory | | 80.50 | 4617 | 4742 | 3818 | 799 | 195 | 4.10 | 244 |
| 7 | | | 72.80 | 4617 | 4700 | 3422 | 1195 | 272 | 4.40 | 265 |
| 8 | Labora- | 476 | 32.38 | 4531 | 4769 | 3928 | 603 | 176 | 3.42 | 119 |
| 9 | tory | 472 | 79.27 | 4478 | 4688 | 3715 | 756 | 207 | 3.64 | 155 |
| 10 | | 472 | 66.50 | 4634 | 4527 | 3010 | 1624 | 335 | 4.85 | 170 |
| 11 | | 81 | 33.92 | 4531 | 4766 | 3999 | 532 | 161 | 3.30 | 103 |
| 12 | | 81 | 72.93 | 4634 | 4657 | 3395 | 1239 | 271 | 4.58 | 170 |

since it is this information which is required for calculations of the heating and ventilating equipment in the design of industrial enzyme plants.

## EXPERIMENTAL

The work was carried out under industrial conditions. For comparison the fungus in some experiments was cultured in conical flasks plugged with cotton stoppers.

The total amount of heat liberated by the fungus during its whole cycle of growth was determined as follows. We measured accurately the quantity of bran used in preparation of the nutrient medium, the water content of the bran, and the dry weight of the fungal culture obtained including the remains of the nutrient (yield of technical preparation).* The calorific values of the bran and the preparation were determined in a calorimetric bomb. From the difference (calorific value of bran minus the calorific value of the technical preparation multiplied by its yield) we found the amount of heat liberated during the whole cycle of growth of the fungus.

These results, in terms of 1 kg of absolutely dry initial bran, are shown in the table. For the calorimetry we deliberately chose cultures from different experiments which gave different yields of technical preparation.

The plant experiments were conducted with an industrial strain (No. 476-I) of A. oryzae and in the laboratory experiments we used other strains as well, No. 81 and No. 476. In addition to the total heat evolution during the whole cycle of fungal growth (column 7), the table gives the data indicating the amount of heat liberated from 1 g of the nutrient substances involved in the biological process but not assimilated by the fungus, i.e., subjected to catabolism (column 9), and also the enzymatic activity of the preparations in units of amylolytic power ("AP" units).†

From an examination of the data in the table we see that the amount of heat liberated per kg dry bran of initial nutrient during the whole cycle of growth varies considerably with the yield of the preparation. The lower the yield of preparation, i.e., the greater the amount of nutrient substances used for catabolic processes, the greater is the heat evolution. For a normal industrial yield of 77–80% the heat evolution is 960–980 kcal, i.e., during the whole cycle of growth an average of 44–45 kcal of heat are liberated per hr per 1 kg of dry bran.

If the results of the laboratory and plant experiments are compared, a fairly distinct tendency can be noted. When the yields of the preparation are similar (experiments 3 and 6, 3 and 9) the heat evolution under industrial conditions is higher than in the laboratory. The only apparent explanation for this is that in industry the culture vessels were well ventilated whereas in the laboratory experiments, the air exchange was reduced to a minimum due to flask culture. The increased oxygen supply to the growing culture obviously leads to more far-reaching oxidative processes, resulting in a greater evolution of heat. This is also indicated by the higher coefficients for heat evolution per kg of substances consumed in industrial conditions as compared with the laboratory experiments. On the other hand, a typical feature of both the plant and laboratory experiments was the increase of these coefficients with the fall in yield of the enzyme preparation, i.e., with increase in the consumption of nutrient substances. This is obviously because the fungus at first consumes the substances which it can utilize most fully in its synthesizing activity, primarily carbohydrates. The greater part of their energy

*Here and later in the paper the term "enzyme preparation", in accordance with RTU UKr. SSR 45–58, means the dried A. oryzae culture with the remains of the unused nutrient.

†Determined by the method adopted in RTU UKr. SSR 45–58 for an enzymatic preparation of A. oryzae.

is assimilated into mycelium and less is converted to heat. Later, as the medium become impoverished in these substances, others, such as proteins and fats, which are less effective for synthesis from the viewpoint of energy, become more involved in the biological process. While their energy potential is greater on the whole, the degree of utilization of their energy in the process of synthesis is less; thus a greater amount is released as heat. It is also worth noting that for similar yields of enzyme preparation the heat evolution is practically independent of the strain of fungus employed (e.g., experiments 6 and 9, 7 and 12).

It is of interest to note that the amylolytic power usually increases as the yield of the preparation falls.

In the study of the dynamics of heat evolution we adopted another method, one which is usually employed in heat engineering studies. In essence it consists in measuring the amount of air used for removal of the evolved heat and the heat content of the air at the inlet and outlet of the culture vessel. The quantity of air was measured by an anemometer and the corresponding values of the heat content of the air were found from a J/D diagram or special tables on the basis of the readings of a dry-bulb thermometer and the relative humidity of the air. The heat evolved was calculated from the difference in heat contents of the air at the outlet and inlet with allowance for its quantity. This method enabled us to carry out the necessary measures and determinations at any time without disturbing the biological process.

Sixteen experiments on cultivation of the fungus were conducted under industrial conditions by this method. At air velocities less than 1 m/sec (less than 8.6 $m^3$ air per hour per kg of bran) the rate of heat removal was obviously inadequate. The culture, especially in the last trays in the course of the air flow, became very overheated, growth was inhibited, and the enzymatic activity of the preparation was low.

Normal growth of the fungus and preparations with high enzymatic activity were not obtained until the air speed reached 1.5–2.0 m/sec, i.e., an air supply of 13–18 $m^3$ per hr per kg of bran.

The results, illustrating the dynamics of heat evolution in kcal per hr per kg of air-dried bran (humidity 12%) for this typical industrial case with normal industrial yields (77–81%), are shown as curves in Figs. 1, 2, and 3. The value Q on the graphs expresses the total heat evolution during the whole cycle of growth of the fungus. The graphs reveal that the evolution of heat by the fungus becomes appreciable 5–6 hr after inoculation and rapidly reaches a maximum by 16–18 hr of growth, after which it falls off sharply. At the peak, 80–90 kcal of heat are released per hr per kg of mold bran. The value determined by this method for the total heat evolution during the whole cycle of growth of the fungus, for similar yields of preparation (77.4 and 77.0%), practically coincided with that found by calorimetry: 943 and 986 kcal/kg of bran, respectively (see values of Q in expt. 4 in table and in Fig. 1).

These experimental data provide the necessary basis for accurate calculations for the ventilation plant and the appropriate air heaters in the design of workshops and factories for enzyme production so as to provide efficient ventilation of the culture chambers. The following points established by experiment and verified in the plant must be taken as a guide.

The temperature of the air supplied to the culture vessel in the peak period of heat evolution must not

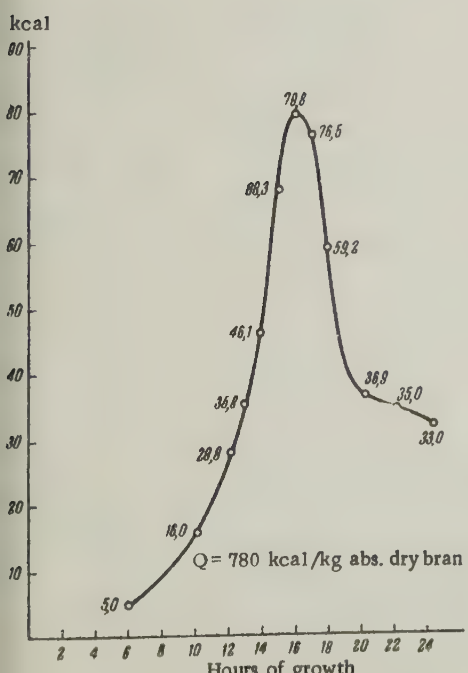


Fig. 1. Dynamics of heat evolution in kcal per hr per kg of air-dried bran.

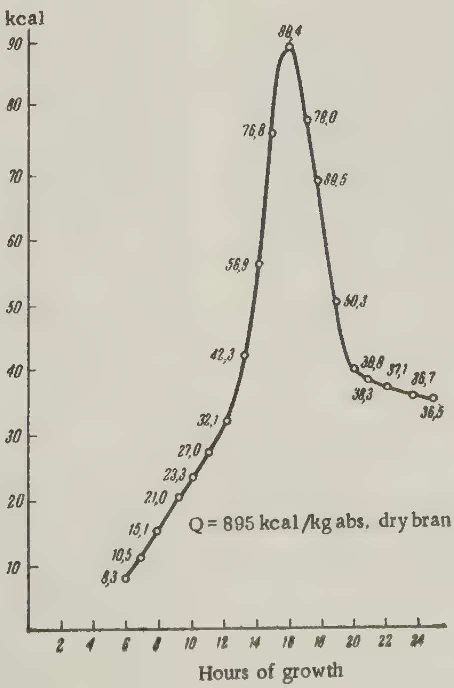


Fig. 2. The same.

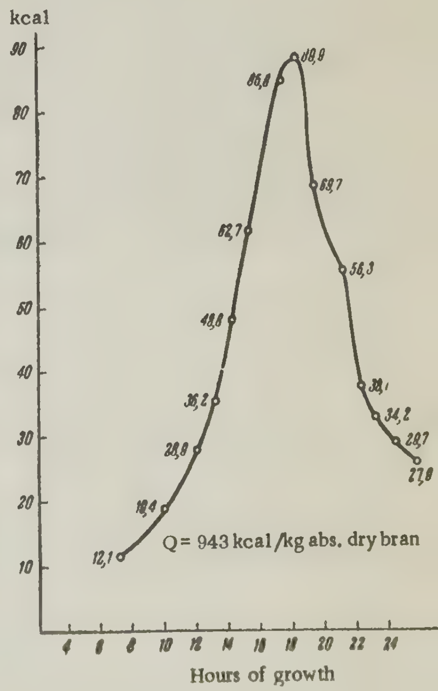


Fig. 3. The same.

be below 28° C; otherwise, the first trays in the air stream are overcooled and the growth of fungus in them is retarded.

Drying up of the nutrient has a very adverse effect on enzyme preparation; therefore the air delivered to the culture chamber throughout the whole cycle of growth must be completely saturated with water vapor (relative humidity 100%).

The temperature rise of the air due to the excess of physiological heat removed from the culture vessel must not exceed 2-3° C.

Hence if air at t=28° C and r.h.=100% is supplied to the culture vessel in the peak period of heat evolution and if the issuing air is heated to 31° C and is still completely saturated with water vapor, its heat content at the inlet will be 21.84 kcal/kg and at the output 25.55 kcal/kg. Each kg of air used for ventilation will thus remove 3.71 kcal of heat. For removal of 90 kcal of heat, 90: 3.71≃24 kg or about 20 $m^3$ of air per hr will be required for every kg of mold bran in the peak period of heat evolution. A prolonged test under plant conditions fully confirmed this. A supply of 20 $m^3$ of conditioned air per hr per kg of bran at the stage of peak heat evolution must therefore be adopted as basic in the design of commercial enzyme plants.

In providing efficient ventilation for the culture vessels it must be borne in mind that the oxygen required for the physiological needs of the fungus can be obtained from a much smaller quantity of air than is required for excess heat removal. A theoretical calculation shows that for this purpose a little more than 1 $m^3$ of air is required per kg of bran throughout the whole cycle of fungal growth. However, ventilation of the vessels even by fully saturated air, because of its heating during passage between the trays, inevitably results in the removal of moisture and the drying up of the nutrient medium, thus adversely effecting the enzyme-producing capacity of the fungus, as mentioned.

Hence the rate of ventilation of the vessels must be coordinated with the heat evolution curve. In the first 8-10 hr following inoculation, when the heat evolution is low, a supply of 3-5 $m^3$ of air per hr for each kg of bran load is adequate. To prevent overcooling of the nutrient at this stage the saturated air delivered to the vessel should have a temperature of 30-32° C. Later, as the evolution of heat increases, the amount of air must be increased and its temperature gradually reduced to 28° C. In the 15-19th hours of growth the ventilation rate must be highest and after this it is again reduced. A mist must be maintained in all parts of the culture vessel.

## SUMMARY

1. The mold Aspergillus oryzae, used in the enzyme industry, liberates a great amount of heat into the surrounding medium during its growth. This heat must be removed from the culture vessels; otherwise the resultant overheating of the culture causes a marked reduction of the enzyme-producing capacity of the fungus.

2. The total heat evolution and the dynamics of this process in industrial conditions were determined experimentally by cultivating the mold on a wheat bran medium. The total heat evolution per kg of mold bran for 24 hr of growth and a normal enzyme yield (77-80%) was 960-980 kcal. Heat becomes appreciable 5-6 hr after inoculation and rapidly rises to a maximum in the 16-18th hour of growth. In the peak period as much as 90 kcal of heat are released per hr from each kg of bran mold.

3. The removal of excess physiological heat by ventilation of the culture vessels under industrial conditions requires the delivery of about 20 $m^3$ of conditioned air (t=28-29° C, r.h.=100%) per kg of bran load to the vessel during the peak period of heat evolution. The rate of ventilation of the culture vessels must be coordinated with the heat evolution curve.

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# THE OCCURRENCE OF SCHIZOSACCHAROMYCES POMBE AT HYDROLYSIS PLANTS

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In 1949, in the fermentation vats of the alcohol sections of hydrolysis plants, a new alcohol producer appeared, of the genus Schizosaccharomyces, similar to the agent of industrial alcoholic fermentation in southern countries.

In the temperate climate, other species of the genus Schizosaccharomyces have been found in fruit and berry juices (Shcherbakov and Popova, 1934; Chalenko, 1941), confectionary bulk (Nepomnyashchaya and Rubanovich, 1938), and in fruit paste (Stadnichenko, 1940).

At first, these yeasts appeared in the vats of the Khor hydrolysis plant in the Khabarovsk region (Kryuchkova and Fisher, 1951), and then at the Kan plant in the Krasnoyarsk region (Ochneva, 1956).

Next, within a short period of time (1954-1956), these yeasts populated the fermentation vats of the Segezha (Karelo-Finnish SSR), the Kos'va (Central Ural), and the Arkhangel'sk hydrolysis plants (Vodolazova et al., 1958), completely displacing active local races of Saccharomyces cerevisiae which the plants had used for fermenting hydrolyzates by the continuous method over a period of many years.

This replacement of one yeast by another went on under plant conditions during five to seven months. At the same time, the schizosaccharomycetes slowly accumulated to a concentration of 40-50%, while the saccharomycetes exhibited all the signs of inhibition: thickening of the cell wall and decrease in dimensions, coarsely granular structure of the protoplasm, increase in percent of dead cells, and the agglutination of many cells into flocs and precipitation as a sediment.

Simultaneously with the appearance of the new yeasts in the fermentation vats, an increase in alcohol yields from 100 kg of fermented SE (soluble extract) was noted at all of the indicated plants. Thus, at the Khor plant, on cedar hydrolyzates, the yield increased from 52.5 to 58.7 liters, while at the Kan plant, on pine hydrolyzates—from 50.1 to 56 liters; the same thing took place at the rest of the plants.

The spontaneous appearance of this genus of yeast was also noted at other hydrolysis plants—the Leningrad and Saratov plants.

In the latter cases, the accumulation of schizosaccharomycetes in fermentation vats reached 5-15%, and then they were observed to die off rapidly.

At the Leningrad plant, these yeasts appeared several times and always died off without reaching a more significant concentration in the fermentation mixture.

Schizosaccharomyces were isolated into pure cultures from the fermentation mixtures from those plants where they became the production culture and were responsible for a high yield of alcohol, and from plants where their increase did not go beyond 5-15%.

All of the cultures were found to be similar to one another according to all characteristics, and could be related to Schizosaccharomyces pombe. However, their behavior toward galactose was different.

This was determined in the following manner.

Two hundred ml of a 2% galactose solution was placed in flasks into which 3% yeast autolyzate wws added. The fermentation of galactose was continued for six days at 30°. The schizosaccharomycetes were compared with the production race of Saccharomyces cerevisiae Leningrad 33, which ferments galactose.

At the end of the fermenation, sugar and alcohol were determined in the fermentation mixture (Table 1).

As seen from Table 1, the Khor and Kan races of schizosaccharomycetes fermented galactose more completely and produced larger quantities of alcohol than Saccharomyces cerevisiae Leningrad 33 under identical conditions of cultivation.

During the course of six days, the remaining schizosaccharomycete races carried out negligible fermentation which had no industrial significance.

Thus, the advantage of active races of Schizosaccharomyces pombe as compared with low-activity races of this genus and with active alcohol races of Sacch. cerevisiae is their ability to vigorously ferment galactose, which constitutes nearly 10% of the hexose sugar of wood wort.

This property, along with others (Drublyanets et al., 1929), enables the schizosaccharomycetes to develop more actively than other alcohol yeasts in the fermentation vats of the hydrolysis plant.

At two of the hydrolysis plants—the Khor and the Kan plants, where fermentation of hydrolyzates was carried out entirely by schizosaccharomycetes—the distribution of these yeasts was studied in all parts of the plants through which cooled wort or fermentation mixture ran. The air of these divisions was also investigated.

Samples were inoculated on malt agar. In order to detect yeast microflora in the air, sterile Petri dishes with malt agar were opened for three minutes.

Data on the content of yeast microflora are presented in Table 2.

Table 1. The Characteristics of Active and Low-Activity Races of Schizosaccharomyces pombe with Regard to Galactose Fermentation (The initial medium obtained 1.73% galactose; pH of medium, 7.0)

| Yeast | Duration of fermentation in days | After fermentation | | |
|---|---|---|---|---|
| | | pH | galactose in % | alcohol in volume % |
| Sacch. cerevisiae | | | | |
| Leningrad 33 race | 6 | 5.4 | 0.04 | 1.01 |
| Schizosacch. Pombe | | | | |
| Khor race | 4 | 5.0 | 0 | 1.11 |
| Kan race | 4 | 5.2 | 0.04 | 1.06 |
| Leningrad race | 6 | 5.2 | 1.38 | 0.14 |
| Saratov race | 6 | 5.2 | 1.64 | 0.07 |

Table 2. Distribution of Schizosaccharomycetes in the Hydrolysis Plant

| Place where sample was taken | | Khor plant | | Kan plant | |
|---|---|---|---|---|---|
| | | total number of yeasts in thousands/ml | schizo-saccharo-mycetes among them, in % | total number of yeasts in thousands/ml | schizo-saccharo-mycetes among them, in % |
| Plant yard: | air | 0 | 0 | 0 | 0 |
| Cooler: | air | 20 | 10 | 37 | 0 |
| | racks | 1230 | 30 | 1720 | 0 |
| | wort | 126 | 30 | 48 | 0 |
| Filter-press: | air | 115 | 0 | – | – |
| | wort | 60 | 0 | – | – |
| Heat exchangers: | wort | – | – | 0 | 0 |
| Wort collector: | wort | 150 | 10 | 138 | 0 |

Note: The sign — designates that the determination was not made.

As can be seen from Table 2, in the course of preparing wood wort, it is infected by yeast microorganisms, but at the Kan plant, schizosaccharomycetes were never found among them; at the Khor plant, however, they were present in samples from the cooler and in the wort collector.

This is explained by the fact that, first, yeast sediments from the fermentation vats accumulated near the cooler, and second, when the fermentation battery was filled too full, the fermentation mixture partially spilled over into the wort collector which was located at the upper level of the fermentation vat.

It is important to note that the spontaneous onset of fermentation of average daily wort samples in the laboratory and of considerable reserves in the fermentation division occurs with the participation of saccharomycetes alone, despite the fact that schizosaccharomycetes occupy the dominant position in fermentation vats.

At both plants, the fermentation of hydrolyzate to alcohol was carried out by the continuous method in two-member batteries with a yeast content of 20-25 g/liter.

The ratio of schizosaccharomycetes to saccharomycetes, as well as their condition, is presented in Table 3.

The principal microflora of the fermentation vats at these plants belongs to the schizosaccharomycetes, which constituted 98–99% of the total amount of yeast. The saccharomycetes, especially live cells, were of negligible importance—1.5-2.0%.

Despite the high concentration of yeast in the fermentation mixture attained in the process of fermentation without special cultivation, the content of dividing schizosaccharomycetes was low—4-5%. Dividing cells rapidly separated from one another, and under the microscope, the process of division could be seen only at the early stages.

Schizosaccharomycete cells in wood fermentation mixture are morphologically heterogeneous.

The most frequently encountered forms are long cylindrical cells with rounded ends; as a rule, one end is blunt, while the other is slightly pointed. Cells with one end greatly widened are encountered in considerable numbers. Sometimes there is a cross-wall immediately beyond the widened end and the cell appears to be divided into two unequal parts. Sole-shaped cells are occasionally encountered.

At the bottom of the fermentation vats, yeast sediments accumulate which consist primarily of dead schizosaccharomycetes and saccharomycetes. The latter are grouped in large flocs, sometimes occupying the entire field of vision.

The high percentage of dead schizosaccharomycetes—55-60%—in fermenting wort should be noted. At all plants where fermentation of hydrolyzates is carried out by schizosaccharomycetes, the number of dead cells is also considerable; however, the alcohol yield remains high and constant.

The large number of dead cells in actively fermenting vats is explained by the fact that these yeast

Table 3. The Quantitative and Qualitative Composition of Yeasts in Fermentation Vats

| Place where samples were collected | Schizosaccharomycetes | | | Saccharomycetes | | |
|---|---|---|---|---|---|---|
| | no. of cells millions/ml | live, % | dividing, % | no. of cells millions/ml | live, % | budding, % |
| Khor plant | | | | | | |
| head vat | 259.5 | 42.2 | 4.3 | 4.2 | 4.7 | 0 |
| tail vat | 339.0 | 46.0 | 5.4 | 9.0 | 0 | 0 |
| Kan plant | | | | | | |
| head vat | 243.0 | 43.2 | 5.2 | 3.5 | 4.2 | 0 |
| tail vat | 316.7 | 40.5 | 4.1 | 4.2 | 4.7 | 0 |

are very sensitive to changes in culture conditions (Drublyanets et al., 1959). Interruptions of several hours in the supply of wort to the fermentation vats markedly worsens their condition; large granulation appears which diffracts light sharply, the number of vacuoles increases, and fermentation energy is reduced.

Increases in acidity and temperature of the wood wort, even for a short time, also lead to a noticeable increase in the number of dead cells in the fermentation mixture.

Part of the dead cells get into the fermentation mixture from the bottom of the fermentation vats. In distinction from saccharomycetes, which give a very thick sediment on the bottom of fermentation vats, yeast sediments made up of dead schizosaccharomycetes are easily suspended; they become turbid when a strong stream of wort is supplied to the fermentation vat.

The investigation of hydrolysis plants in order to determine the distribution of schizosaccharomycetes in them showed that these yeasts develop profusely only in the course of fermentation, and only here are capable of replacing saccharomycetes.

They do not develop at any other stages of preparation of wood wort or during its storage in the laboratory and in the fermentation divisions.

The question of whether schizosaccharomycetes occur near hydrolysis plants in residential settlements, in household products and half-finished products, and in public eating establishments was of great practical and theoretical interest. A large number of samples of wheat and rye dough, leavened dough, liquid bread yeasts, pressed and dried yeasts, beer, and kvass were collected. Thorough microscopic examination and inoculation on liquid and agar-containing malt wort was carried out on all samples obtained.

No schizosaccharomycetes were found in a single one of the samples; only budding yeasts were growing everywhere.

A search for schizosaccharomycetes was also carried out in the fermentation industries at Khabarovsk with which the Khor plant is associated in its practical activity.

A study of the microflora of the alcohol and beer-brewing plants provided convincing evidence that the industrial fermentations there are carried out by saccharomycetes; no schizosaccharomycetes were encountered even in the form of single cells.

In the neighborhood of the Kan plant, only food products and half-finished products (dough, leavened dough, beer, yeasts) were examined. The growth of budding yeasts was observed in all samples.

The circumstance that Schizosacharomyces pombe grew spontaneously at hydrolysis plants situated in different parts of the USSR suggests that wood hydrolysates are a favorable medium for them.

Under these conditions, they proved to be active agents of industrial alcoholic fermentation. We did not find this species in other fermentation industries near hydrolysis plants. Aside from wood wort, other media which are favorable for its growth are fruit and berry juices (Shcherbakov and Popova, 1934; Chalenko, 1941), fruit paste (Stadnichenko, 1940), and confectionary bulk (Nepomnyashchaya and Rubanovich, 1938). What specific factors convert the indicated media into a habitat for this microbe still remains unclear.


SUMMARY

1. It has been established that, at some hydrolysis plants, the yeast Schizosaccharomyces pombe completely replaces Saccharomyces during the course of 5-7 months, while at other plants, the proliferation of these yeasts only reaches 5-15%.

Strong races of Schizosaccharomyces pombe from those with low activity in their behavior toward galactose; the former carry out active fermentation of this carbohydrate with good yield of alcohol.

2. The occurrence of schizosaccharomycetes at the plant was observed only in fermentation vats.

3. Schizosaccharomycetes were not encountered in fermentation industries situated near hydrolysis plants.


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# THE USE OF PAPER CHROMATOGRAPHY IN THE CLASSIFICATION OF ACTINOMYCETES

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Because of the increased significance of actinomycetes as producers of antibiotics and other metabolites, there is a growing demand for their grouping and classification as well as a necessity for developing rapid and reliable methods for recognition of freshly isolated and studied cultures. We believe that a species of actinomycete may be defined only from the sum total and not from one or two characteristics, regardless of the constancy of the latter. However, every characteristic employed for this purpose must be carefully investigated.

Recently the technique of paper chromatography of different compounds, including antibiotics, has become widespread. With the help of paper chromatography it is possible to separate highly complex mixtures of similar compounds; e.g., it has been shown that crystalline actinomycins consist of several components. Because this method is quite simple and accessible to any laboratory its use has increased. Furthermore, only extremely small concentrations of the tested materials are required for its application. Ishido et al.,* Takaaki (1955), Shevchik (1959), and others used paper chromatography for separation and identification of antibiotics. By means of this method and utilizing eight different solvents, Takaaki separated seventeen antifungal antibiotics produced by actinomycetes into seven groups. He demonstrated some similarity between certain antibiotics, e.g., actidione and fermecidin, etc. However, it must be pointed out that in grouping of the above antibiotics no correlation has been observed with other characteristics, such as diffusion curve, UV absorbtion spectrum, and antifungal spectrum.

Shevchik, utilizing micromethods for isolation of new and unknown antibiotics, suggests the use of ten different solvents. The author showed differences in solubility of two antibiotics, streptomycin and an unknown antibiotic BU607, isolated in his laboratory. On the basis of his data he concluded that it is possible by use of paper chromatography to demonstrate and determine the solubility of an unknown antibiotic independently in a culture medium. To illustrate the results of his experiments, Shevchik used distinctly different antibiotics, streptomycin and antibiotic BU607; the latter, judging from the included chromatogram, being closely related to actinomycins.

Betina (1959) used solvents at different pH for differentiating one antibiotic from another by means of paper chromatography. This allowed him to evaluate the characteristic of the antibiotic (acid, basic, or neutral) without isolating it from the culture medium. These investigators applied the method of paper chromatography for identification or differentiation of freshly isolated antibiotics without considering the species of the product.

The purpose of the present study was to determine the possibility of utilization of paper chromatography for separation of actinomycetes producing antibiotics.

## METHODS

The method of distributing ascending paper chromatography was used. Chromatographic paper labeled "slow" and manufactured by a Leningrad factory was cut into strips 1 cm wide and 20–50 cm long, depending on the containers used in this process. For the latter we utilized cylinders of 20–50 cm height and 3–4 cm in diameter. An agar block of an actinomycete culture of 0.5 cm diameter was placed on a strip of chromatographic paper 4 cm from the end, with mycelium away from the paper. For analyzing culture media 0.1 ml was applied by means of a micropipette. Our results showed that the best results were obtained from the agar blocks. Experiments were conducted as follows: A culture of the actinomycete was grown on agar medium for 5–7 days at 27–28°C. The most suitable medium for production of antibiotic substances was selected in advance. Usually a fish or soybean medium was used. The composition of the fish medium was (in %): fish extract, 1–1.5; glucose, 2; chalk, 0.35. The composition of the soybean medium (in %): soybean flour, 2; glucose, 1.5; NaCl, 0.1; chalk, 0.3. In all media 1.5% agar was used.

Five to seven days after cultivation of an actinomycete on one of the above media, blocks were cut out and placed on chromatographic paper. In order to increase the concentration of antibiotic, fresh blocks were repeatedly placed on paper. Blocks applied at the beginning remained on paper until completely dehydrated, when they were removed and replaced by new blocks. Subsequently the strips of paper were hung from a stopper of a cylinder by means of glass hooks or by another method, and 0.5–1 cm of the free end was immersed into the solvent. Deposited substances (applied blocks) must be located 1–2 cm above the solvent. The paper, placed vertically into the solvent must be level and without creases. After positioning

*Quoted from Takaaki (1959).

the paper in the solvent, the cylinder is sealed for 10–12 hr until the solvent reaches the required height on the strip. Then the strips are removed from the cylinders and dried in air or by a fan. The dried strips of chromatographic paper are placed on agar medium inoculated with an indicator organism and are incubated at a temperature optimum for the growth of the organism. Lack of growth of the indicator organism, i.e., zones of inhibition, locate the antibiotic substance on paper. In order to evaluate the similarity of the tested antibiotic with already established antibiotics, the latter are used as controls. Since in our study we used native antibiotics without separating them from their nutrient medium, we also used native antibiotics produced by known species of actinomycetes as controls. At first we determined $R_f$ values for our chromatograms; however, since our investigations were carried out with impure antibiotics, the characteristic regularity of distribution was not clearly established on paper and we discontinued the determination of this factor, simply indicating the distribution of antibiotic spots from the zone of their localization in agar (Fig. 1).

We also used circular or radial chromatography in our studies. This method as outlined below has an advantage over ascending chromatography inasmuch as it greatly shortens the time for analysis and allows several tested agents to be located simultaneously on a disc. A Petri dish of 18–20 cm diameter is used (one can also use conventional dishes of 8–9 cm). One selects a cover for the dish so that the two have identical diameters. A solvent is poured into the dish and a circle of chromatographic paper is cut according to its dimension; agar blocks or drops of culture fluid are placed on a disc 1 cm from the center in a manner similar to that used for ascending chromatography. After drying, a tightly wound wick made from chromatographic paper or cotton is placed in a very small opening prepared in the center of the disc. Such a circle of chromatographic paper with the tested preparations is placed on the edges of the cup with the wick dipping into the solvent. The cover is placed on the dish with the paper circle pressed between the edges. The migration of the compounds is complete within 1–4 hr. Migration paper discs are then dried and radial strips of paper are cut out and developed in agar inoculated with an indicator organism. As stated above, it is not possible to place the entire circle on agar since antibiotics diffuse from paper into agar and run into each other, resulting in an indefinite runny zone. Controls are also used here for comparison. It is thus possible to separate antibiotic substances in the samples under investigation and determine their respective solubility in different solvents.

In the course of our investigations we tried many solvents as well as mixtures or systems of solvents and concluded that the solvents proposed by Shevchik (1959) are more or less suitable for our purposes. We used the following group of solvents: (1) water, (2) 20% $NH_4Cl$, (3) methanol, (4) acetone, (5) ethyl acetate, (6) butanol, (7) thio-ether, (8) chloroform, (9) benzene, (10) petroleum ether.†

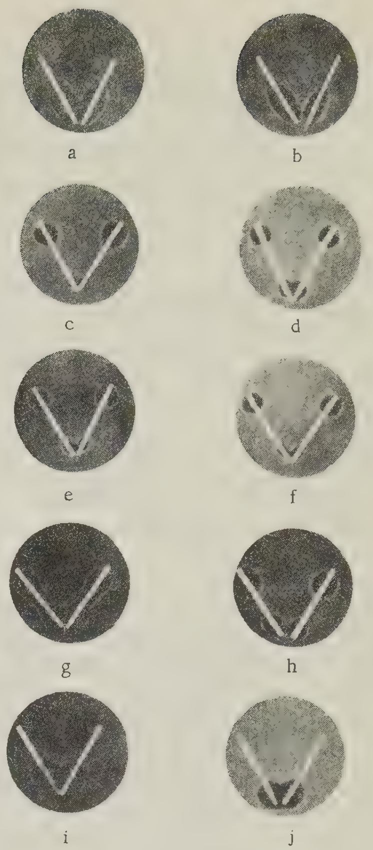


Fig. 1. Actinomyces flaveolus 1015B in different solvents (circular chromatography). Indicator organism—Staphylococcus aureus. The actinomycete was grown on fish medium for 7 days. Zones indicate the position of investigated antibiotic. a) Water; b) 3% $NH_4Cl$; c) methanol; d) acetone; e) ethyl acetate; f) butanol; g) thio-ether; h) chloroform; i) benzene; j) petroleum ether.

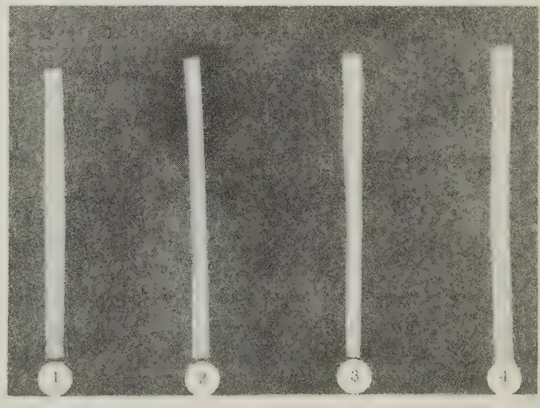


Fig. 2. Antibiotic compounds produced by actinomycetes secreting actinomycins in a solvent of 20% $NH_4Cl$ on indicator organism Saccharomyces cerevisiae. 1) A. 41E sp.n.; 2) A. citreofluorescens 2292; 3) A. chrysomallus 2820; 4) A. flaveolus 1015B.

†This method of numbering solvents will be observed in all tables dealing with chromatograms in different solvents.

| Name of culture | Nature of sporangia | Growth on medium 1 | | | Growth on Czapek's medium with glucose | | | Growth on MPA | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | color of aerial mycelium | color of colonies | color of medium | color of aerial mycelium | color of colonies | color of medium | color of aerial mycelium | color of colonies | color of medium |
| Act. chrysomallus 2820 | straight | pale yellow | greenish brown | yellowish green | pale yellow | brownish | yellowish lemon | pale yellow to yellow | yellow | yellow |
| Act. citreofluorescens 2292 | " | " | " | " | " | " | lemon yellow | " | " | " |
| Act. fluorescens 592 | straight, short | cream yellow to pale yellow | pale yellow greenish | pale cream-yellow | cream | cream-yellow | " | cream yellow | " | light yellow |
| Act. flaveolus 1015 B | spiral | white and yellowish, feathery | yellow | yellow | pale cream | yellowish brown | brown, greenish yellow | without mycelial action | " | not pigmented |
| Act. 41E sp.n. | " | dense pale yellow, mealy | brownish | not pigmented | yellowish white | reddish chocolate | colorless | " | colorless | " |

Legend: ++++ very good growth; +++ good growth; ++ satisfactory growth; — no growth.

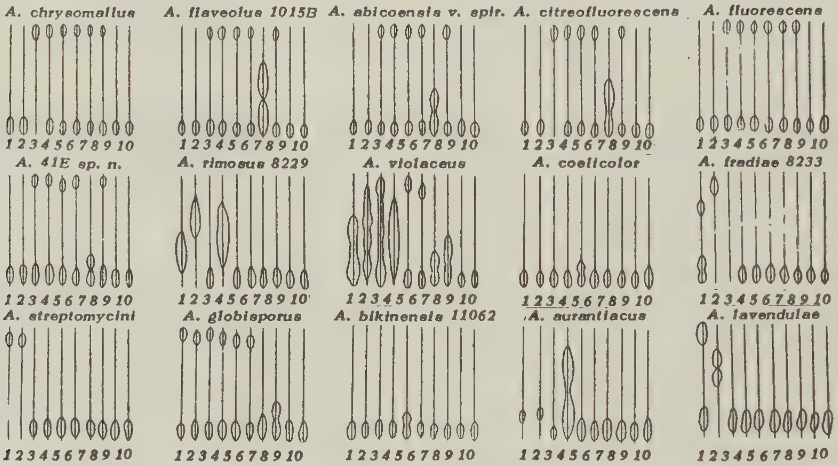


Fig. 3. Combined chromatogram of different actinomycetes grown for 7 days on fish medium. Indicator organism—S. aureus.

It must be pointed out that distribution of antibiotics on chromatographic paper is not always clearly established in one trial despite the simplicity of the procedure. One must repeat an experiment 2–3 times in order to obtain reliable results.

We studied a large number of cultures of actinomycetes, apparently belonging to different species, by means of the indicated method in order to determine the possibility of application of paper chromatography to investigation of characteristics of species of actinomycetes, the producers of antibiotics.

Antimicrobial activity of actinomycetes in relation to several indicator organisms was checked every time prior to placing of the actinomycete blocks on chromatographic paper. This allowed correct selection of indicator organisms for evaluation of the distribution of antibiotics on paper. Experience has shown we cannot limit ourselves to one indicator organism, inasmuch as the actinomycete in question may produce several antibacterial and antifungal agents. In developing a chromatogram using Bacillus subtilis or S. aureus, we discover only the antibacterial antibiotics which may be present in cultures of different species, e.g., A. chrysomallus and A. citreofluorescens. They produce actinomycin "C" but they are differentiated in that A. chrysomallus does not form a water-soluble anti-yeast antibiotic, which is present in A. citreofluorescens, as ascertained from a chromatogram developed on S. cerevisiae (Fig. 2).

Figure 3 shows a noticeable difference in compounds on chromatograms of widely differing species of actinomycetes such as: A. rimosus, A. streptomycini, A. violaceus, A. coelicolor, A. aurantiacus, A. chrysomallus, etc. All these organisms synthesize different antibiotics. Conversely, differences in compounds on chromatographic paper are absent in actinomycetes producing a common antibiotic. For example, it is not possible to differentiate, by means of paper chromatography, antibiotics of an actinomycete produced in a series of culture media. Even though organisms producing the antibiotics differ markedly from each other, e.g., A. chrysomallus 2820, A. citreofluorescens 2292

that Showed a Similar Uniform Chromatogram

| Antimicrobial spectrum | | | | Interaction | | | | | Assimilation of carbon source | | | | | Formation of actinomycin |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Gram-positive bacteria | Gram-negative bacteria | S. cerevisiae | C. albicans | 2820 | 2292 | 592 | 1015B | 41E | glucose | saccharose | rhamnose | lactose | mannitol | |
| + | − | − | − | − | − | + | − | + | ++++ | − | ++++ | +++ | ++++ | Positive |
| + | − | + | − | + | − | + | − | + | +++ | − | ++++ | +++ | ++++ | " |
| + | − | − | − | + | + | − | − | + | +++ | − | − | ++ | +++ | " |
| + | − | − | − | + | + | + | − | + | ++++ | − | − | − | − | " |
| + | + | + | + | + | + | + | + | − | ++++ | +++ | + | − | − | Not established |

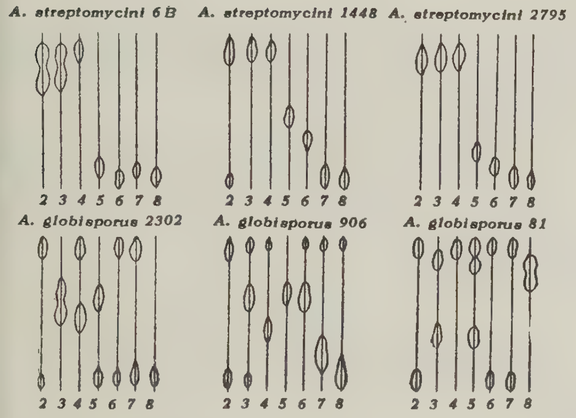


Fig. 4. Chromatogram of water-soluble substances produced by A. streptomycini and A. globisporus using phosphate-citrate buffer at varying pH.

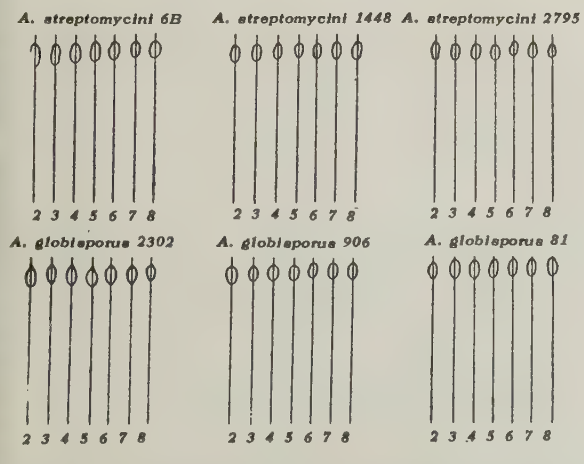


Fig. 5. Chromatogram of water-soluble antibiotic compounds produced by A. streptomycini and A. globisporus in 10% $NH_4Cl$. The paper has been previously treated with phosphate-citrate buffer at varying pH.

A. fluorescens 592, and even though they unquestionably belong to different species, they produce 2–3 antibiotics, one of these being common to the type of actinomycete. The others are different and are characteristic of the individual organisms. Shoule one prepare a chromatogram for the unmasking of the antibiotic common to all these species it becomes impossible to differentiate between them. Similar results are obtainable in comparing the substances in A. flaveolus 1015B, A. abicoensis var. spiralis,‡ and A. 41E. They differ from the above-indicated three examples by having spiral sporangia. The essential characteristics used to differentiate these species are summarized in Fig. 3.

According to Betina's instructions we used solvents at different pH. We were able to differentiate certain antibiotics utilizing phosphate-citrate buffers of varying pH. The antibiotic produced by a culture of A. streptomycini, i.e., streptomycin, appears as a single substance of acid nature, while a water-soluble antibiotic produced by A. globisporus forms several spots on paper at different pH (Fig. 4).

It is apparent from the above-indicated scheme that one can utilize the method in question for differentiation of antibiotics and that pH is unquestionably important in distribution of substances on paper. However, one must select the solvent for every experiment carefully and process the paper differently for each compound in question. Otherwise merely changing pH will not produce the desired results. For example, it is not possible to differentiate the two above-indicated antibiotics by treating the paper with acid citrate-phosphate buffer at varying pH and using 10% $NH_4Cl$ as a solvent (Fig. 5).

For us it was important to establish the appearance of combined chromatograms of compounds produced by strains of a single species. Do they yield one and the same chromatogram? The following experiments

‡A. flaveolus 1015B and A. abicoensis var. spiralis (the latter has been received from the Institute for Investigation of New Antibiotics) are synonymous and represent one and the same species.

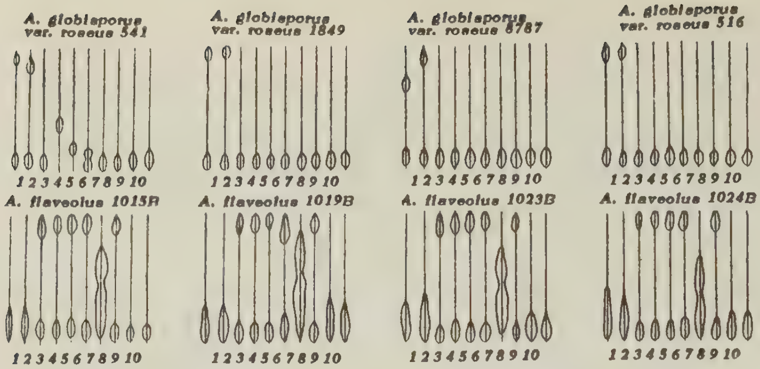


Fig. 6. Combined chromatogram of individual strains of A. globisporus var. roseus and A. flaveolus.

were performed to elucidate this question. The selected cultures of different species consisted of four strains belonging to A. globisporus var. roseus and four to A. flaveolus. Native antibiotics obtained from all cultures were investigated by means of paper chromatography. From Fig. 6 it is clear that all four strains of A. globisporus var. roseus had a similar chromatogram typical of the species; all of the four strains of A. flaveolus also had a similar chromatogram but different from the one for the first species.

These data show that it is possible to differentiate the cultures of actinomycetes with the help of paper chromatography. However this method can be used only in connection with other microscopic and biochemical methods.

## SUMMARY

1. Paper chromatography data may be used only in conjunction with other characters which reveal the relationship between the strains composing a given species.

2. By means of standard chromatograms of known antibiotics it is possible to reveal and differentiate compounds in the cultures tested. However, paper chromatography is of limited value when used for the identification of species.

3. In order to ascertain by paper chromatography the differences between antibiotic compounds produced by an actinomycete culture the chromatogram should be developed on several indicator organisms rather than on a single one.

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**See English translation.

# A NEW METHOD FOR SEPARATING AEROBIC AND ANAEROBIC SPECIES OF MICROORGANISMS

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Considerable difficulties arise in separating aerobes, anaerobes and facultative forms in isolation of pure cultures of microorganisms. Frequently the material being investigated contains many fungi. Their mycelia cover the entire surface of a dish and consequently it is difficult or completely impossible to isolate colonies of other microorganisms.

The method we proposed was previously employed with success in separating facultative bacteria from fungi and we used it subsequently for separating aerobic bacteria from anaerobic or facultative forms. It should be pointed out that the desired result was achieved in most cases in the course of initial inoculation.

The proposed method is as follows. One takes an ordinary bacteriological test tube and a narrow glass tube (3 mm diam) 3 cm longer than the test tube. The edges of the tube are annealed in flame. A cotton plug is rolled around the upper part of the tube. After placing the tube almost to the bottom of the test tube, the plug is moved up or down and is fixed at a desired level in order to effect a good closure of the test tube. Then the tube and the stopper are removed from the test tube, the ends are plugged with cotton, it is wrapped in paper and sterilized separately. Ten ml of a liquid medium is placed in the test tube, which is then sterilized. The test tube and the tube are assembled prior to inoculation (Fig. 1a,b) and with a sterile pipet 3-4 ml of the same medium is added to insure a sufficiently high level, 3-4 cm below the stopper (it is best to determine in advance the total volume of the medium). After removing the stopper and opening the lower end the sterile tube is inserted into the test tube. In this manner the test tube with medium is ready for inoculation. A loop is used for seeding a mixture of aerobic, facultative and anaerobic microorganisms. After opening the test tube and placing it in an inclined position, the edges supporting the stopper and the tube are flamed, and the inoculum is placed near the surface of the liquid (it is best not to immerse the loop deeply or place the tube into the test tube after inoculation). Position of the tube is checked after replacing the stopper and with light pressure is lowered carefully to the bottom of the test tube. Growth of aerobic organisms can be seen on the surface of the medium in the test tube after 1-2 days of incubation, but not on the surface of medium in the tube. In separating milk-souring bacteria from fungi, the former grew well in both test tube and tube after overnight incubation, while the latter began

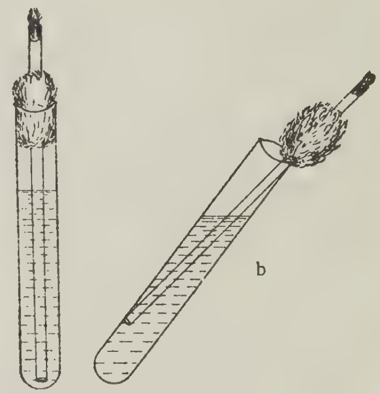


Fig. 1. Test tube assembly. a) Position before sowing; b) position of tube during sowing.

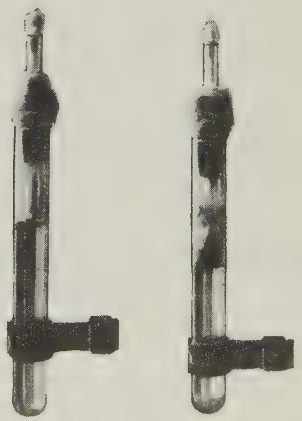


Fig. 2a. A test tube with a tube filled with medium and inoculated with a mixture of facultative and aerobic microorganisms.
Fig. 2b. The same test tube after incubation. A heavy growth of aerobe is seen on the surface; the facultative anaerobe is in the medium of the tube.

| Mixture of microorganisms | Growth of microbes in test tube | Growth of microbes in tube | Result of separation of microorganisms | Growth of aerobe on control medium (transfer from the tube) |
|---|---|---|---|---|
| Streptobacterium plantarum + Aspergillus oryzae | milk-souring fungus | milk-souring | + | wort -- |
| Betabacterium γ + A. niger | milk-souring fungus | milk-souring | + | wort -- |
| Betabacterium γ + Bacillus mycoides | milk-souring, surface film of aerobe | milk-souring | + | MPA -- |
| Betabacterium α + B. mesentericus | milk-souring, surface film of aerobe | milk-souring | + | MPA -- |
| S. plantarum + B. mesentericus | milk-souring, surface film of aerobe | milk-souring | + | MPA -- |
| S. plantarum + B. mycoides | milk-souring, surface film of aerobe | milk-souring, surface film of aerobe | - | MPA ++ |
| Thermobacterium + B. subtilis | milk-souring, surface film of aerobe | milk-souring | + | MPA -- |
| Saccharomyces cerevisiae XII + B. mycoides | yeast, surface film of aerobe | yeast, surface film of aerobe | - | MPA ++ |
| Saccharomyces cerevisiae XII + Penicillium chrysogenum | yeast, penicillium | yeast | + | wort -- |
| Granulobacter pectinovorum + A. niger | bacteria, mycelium | bacteria | + | wort -- |

Legend: + Separation of aerobes and anaerobes achieved; ++ indication of aerobic growth on control medium; - separation not achieved; -- lack of growth on control medium.

to form mycelium in the test tube only after 2–3 days. Mycelium did not develop in the tube even after 15 days of incubation (Fig. 2a,b). Facultative or anaerobic microorganisms are transferred with a loop or needle after opening the small tube at the top (avoid removing the test tube stopper or shaking the contents of the test tube). In this manner we purified many cultures of milk-souring bacteria contaminated with aerobic bacteria and fungi. Even the first transfer gave positive results. Subsequently we mixed pure cultures of milk-souring bacteria and pure cultures of yeasts and fungi Aspergillus niger, A. oryzae, and Penicillium chrysogenum or with aerobic bacteria Bacillus mesentericus, B. subtilis, B. megaterium, and B. mycoides. The results of these experiments are shown in the table, from which it is seen that in all cases, except for the mixtures consisting of B. mycoides, facultative anaerobic bacteria, yeasts, and acid-fast organisms were easily separated after initial inoculation from aerobic microorganisms. Results with B. mycoides were not consistent in nature.

## SUMMARY

A simple method is suggested for separating aerobic and facultative or anaerobic microorganisms contained in a mixture. The method supplements those available for this purpose. It allows carrying out simultaneously an adequate number of parallel tests or many different experiments.

# A METHOD FOR DETERMINING PHOSPHATASE IN MICROORGANISMS

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An area requiring further investigation is the utilization of organic phosphorus compounds by plants. In the opinion of some authors (Merenova, Kuzin, et al., 1955) these compounds can be adsorbed directly by plants. Others feel these compounds must be broken down before their phosphorus becomes available for plant nutrition (Ratner and Samoilova, 1955).

The phosphatases of the root itself as well as those of the microorganisms of the rhizosphere and soil take an active part in the process of organic phosphate decomposition. The phosphatase activity of microorganisms has been studied by a number of authors. McFadyen (1934) showed that Bacillus subtilis is capable of splitting nucleic acid. Kramer and Erdei (1959) demonstrated that under certain conditions the activity of soil enzymes, particularly phosphatase, can indicate how well the soil is provided with nutrient substances. Krasil'nikov and Kotelev (1957) as well as Kotelev (1958) established that many soil microorganisms have phosphatase activity.

New methods for determining phosphatase activity in microorganisms would help clarify their role in the dynamics of phosphates in soil.

A method is described below for determining phosphatase directly in the area of the plant root system as well as a method for simultaneously determining acid and alkaline phosphatases.

## Method for Determining Phosphatase in the Root Zone of Plants

To demonstrate the role of microorganisms in the mobilization of phosphorus from slightly soluble organic phosphates, the possibility of determining phosphatase distribution directly at the site of contact between the very finest root hairs and the adjacent bacteria was considered. For this it was necessary to select a method showing clearly the phosphatase distribution both in bacteria and in the finest root hairs.

We followed Gomori's method (1946a,b) but instead of bacterial smears we placed thin sections of barley roots with root hairs on the slide. The barley was previously sprouted in Hellriegel's liquid medium bacterized with soil suspension from the rhizosphere of barley. The sections were made on the 15th day of growth of the plants. The method follows:

1. Preparation of sections for staining. In order to attach the root sections to the slide a histological technique was employed, viz., fresh egg white is beaten well and put through a paper filter. The filtrate is diluted with an equal amount of glycerol and a thin layer placed on the slide. A thin section of the surface of barley root is placed on the prepared slide and dried in an incubator at 40–50°C.

2. The preparation was fixed by immersing the slide in a dish of acetone for 10 min.

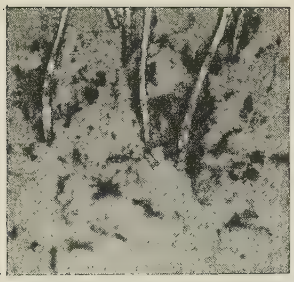
3. To remove soluble phosphates the slides were rinsed in water, immersed in 0.1 M citrate buffer at pH 4.7, and kept in the solution for 30 min. Free soluble phosphates must be removed to avoid a positive reaction later. Citrate buffer was prepared by adding 2 volumes of 0.1 M HCl to 8 volumes of citrate solution (21 g citric acid+200 ml of 1 M NaOH) and water to make 1 liter.

4. For the eventual demonstration of phosphatases, the slides were washed well in distilled water to remove buffer solution and immersed 12 hr in the following substrate: 24 ml of 2% sodium glycerophosphate, 25 ml of sodium barbiturate (Medinal) as buffer in 50 ml of distilled water, 5 ml of 2% $CaCl_2$, and 2 ml of 2% $MgSO_4$. To preserve the solution, several drops of toluene were added. While in the substrate the phosphatases of the preparation act on the glycerophosphate and split off phosphoric acid. In the presence of calcium chloride this immediately precipitates at the site of formation as an insoluble salt, $Ca_3(PO_4)_2$. Magnesium sulfate activates phosphatase.

5. The development of phosphorus compounds. The tricalcium phosphate obtained is a rather transparent, microscopically invisible compound. To make the phosphorus compounds visible the preparation was washed well with water and immersed for 5 min in a 2% solution of cobalt nitrate, yielding an insoluble compound—cobalt phosphate—in an exchange reaction. To improve visibility the preparation was washed well with water to remove free cobalt nitrate and then immersed in a weak solution of ammonium sulfide (2–3 drops ammonium sulfide in 50 ml distilled water). Ammonium sulfide caused an opaque black precipitate of cobalt sulfide wherever cobalt phosphate had been deposited on the preparation. The sites of phosphatase activity were thus clearly visible on the preparation as deposits of black cobalt salt.

The preparations of root sections with the microorganisms surrounding them thus obtained were examined under a BM-3 microscope and microphotographed (figure).

By analysing these microphotographs we concluded that the phosphatase in the root zone of plants is concentrated chiefly in the cells of microorganisms and much less in the root hairs feeding the plants. The



The distribution of phosphatase in the zone of the root system of barley sprouts. The root hairs do not have phosphatase activity; many cells of the root microflora contain phosphatase.

microphotographs also showed that not all microorganisms in the root zone have the same phosphatase activity (some cells were not stained at all). This uneven distribution of phosphatase in microorganisms was also shown in our determinations of phosphatase activity of microorganisms by means of plating soil suspensions on petri dishes.

## The Demonstration of Phosphatases of Microorganisms in Alkaline and Acid Medium

The method we developed earlier for the determination of phosphatase in microorganisms using sodium phenolphthalein phosphate (Kotelev, 1958) makes it possible to determine the total effect of the enzyme by the phenolphthalein released but only in alkaline medium.

With this method it is thus not possible to determine in what medium the hydrolytic action of phosphatases takes place.

King (1943) was the first to synthesize various compounds in which phosphorus was introduced into indicator molecules (bromphenol blue, phenol red, etc.). Through the action of phosphatase these compounds released indicator giving the corresponding color at various pH's of the medium.

For our purposes a preparation of calcium bromphenol blue phosphate was synthesized by a somewhat modified method. This salt has a number of advantages over other similar compounds. It is easily decomposed by the phosphatases of microorganisms and gives a point of color change (pale blue—yellow) at a pH of about 7.

## Method of Making the Preparation

Two g of bromphenol blue is dissolved in 25 ml of dry pyridine in a flask equipped with an electric stirrer. After the indicator is dissolved the flask is well chilled with ice and the solution phosphorylated by the addition of 0.75 ml of freshly distilled anhydrous phosphorus oxychloride. The solution turns a yellow color and precipitation of salt crystals begins.

After the phosphorylation reaction the phenol phosphoryl chloride obtained is decomposed by the addition of 1–2 ml of water. The water is added dropwise and the vessel cooled by ice with constant motion of the stirrer. Then 10–15 ml of alcohol is added to dissolve the crystalline precipitate which falls out, and 7 ml of 40% NaOH and 20 ml of hot concentrated calcium acetate solution (4 g of calcium acetate is dissolved by boiling in 20 ml of distilled water) are added.

A voluminous sediment precipitates. The solution is transferred to a 250 ml conical flask, 200 ml of alcohol is poured in, and the flask is left overnight to precipitate the salt.

The salt is filtered out under vacuum, washed with alcohol and ether, dried, and stored in a sealed container.

The salt obtained is used for the determination of acid and alkaline phosphatase as follows.

Five hundred ml of 0.5% aqueous solution of white agar (Astrakhan, Chinese, or Japanese) is prepared, to which 2.6 g $NH_4Cl$ and 0.2 g $MgSO_4$ are added. The amount of calcium bromphenol phosphate necessary to get a final concentration of 0.2% in the agar is ground in a porcelain or glass mortar. Several ml of water and alcohol are added to the mortar for better grinding. The medium obtained is poured over plates with colonies which had grown on various media (MPA, wort, Czapek's agar, starch-ammonia, etc.). The reaction becomes visible after several hours. A greenish-yellow color usually appears around colonies of fungi, while a blue color appears around many bacteria.

A change in the color indicates that the reagent was decomposed and the released indicator gave the corresponding color due to the pH of the medium.


## SUMMARY

A technique is described for phosphatase assay in the root zone of plants and for demonstration of phosphatases in microorganisms in an alkaline and acid medium.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# SUMMARY OF THE CONFERENCE ON THE PROBLEMS OF CLASSIFICATION AND NOMENCLATURE OF ACTINOMYCETES

**Ya. I. Rautenshtein**

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The problem of classifying the actinomycetes has recently attracted the attention of many investigators. It has been discussed on the pages of microbiological journals and in conferences and symposia, in particular at the Symposium on the Nomenclature of Actinomycetes which took place in Rome in 1953, and also at the round-table conference in August 1958 at Stockholm during the Seventh International Congress of Microbiology. Classification of actinomycetes was the subject of special discussions in the journal "Mikrobiologiya" during 1959 and 1960, in which a number of Soviet and foreign scientists took part.

The growing interest in the problem of classifying the actinomycetes may be attributed to the great practical significance that the actinomycetes possess as producers of the most valuable antibiotics, vitamins, growth stimulators, and other biologically active compounds.

Due to this, the actinomycetes have been intensively studied in many countries of the world, resulting in a sharp increase in the number of described species of these microorganisms and a significant recent expansion in general knowledge of the group.

However, in establishing the new species and in reevaluating the old species various investigators have used different criteria for determining species and have proceeded from different points of view regarding the significance of one or another characteristic for species specificity of actinomycetes.

A characteristic may be considered decisive by one investigator and of secondary importance by another.

In studying and describing new species different investigators have used various media which are not standardized or generally available and, in describing new species, frequently have not conducted comparative studies with previously described forms. Much of the confusion is brought about by failure to conform to the International Code of Microbiological Nomenclature in classifying actinomycetes. The undesirability of this situation is recognized by the majority of scientists working with actinomycetes.

A natural striving to reach an agreement on the basic problems of classifying the actinomycetes to develop a unified method for their study exists.

The editorial board of the journal "Mikrobiologiya" therefore organized a discussion of the problem of classifying the actinomycetes, enlisting the help of the Institute of Microbiology of the Academy of Sciences of the USSR, the Institute for Investigation of New Antibiotics of the Academy of Medical Sciences of the USSR and the All-Union Scientific Research Institute of Antibiotics of the Ministry of Health of the USSR.

The meeting took place in Moscow, June 8–10, 1960, and the following reports were made: 1. Principles of Classifying the Actinomycetes and Rules of Classification of Actinomycetes which Produce Antibiotics, by N. A. Krasil'nikov. 2. The Nomenclature of Actinomycetes in the Light of Contemporary Information on their Genetics, by S. I. Alikhanyan, L. I. Borisova, and L. I. Erokhina. 3. The use of Actinophage in Identifying Actinomycetes, by Ya. I. Rautenshtein. 4. The Diagnostic Significance of Different Characteristics in Classifying Representatives of the Genus Actinomyces, by T. P. Preobrazhenskaya, E. S. Kudrina, M. A. Sveshnikova, and T. S. Maksimova. 5. The Significance of Morphological Characteristics in Classifying Actinomycetes, by E. I. Andreyuk. 6. Comparative Evaluation of Different Nutritive Media for Expression of Morphological and Cultural Characteristics of Actinomycetes, by E. S. Kudrina, T. P. Preobrazhenskaya, M. A. Sveshnikova, and T. S. Maksimova. 7. The use of Electron Microscopy of Spores in Classifying Actinomycetes, by T. S. Maksimova, T. P. Preobrazhenskaya, E. S. Kudrina, and M. A. Sveshnikova. 8. Colonial Form as a Species Characteristic of Actinomycetes, by A. A. Prokof'eva-Bel'govskaya and Z. B. Shamina. 9. Physical Properties as a Taxonomic Characteristic in Classifying Actinomycetes, by V. D. Kuznetsov, N. M. Lugina, and E. I. Sorokina. 10. The Stability of Physiological Characteristics and their Significance in Classifying Actinomycetes, by M. A. Sveshnikova, E. S. Kudrina, T. S. Maksimova, and T. P. Preobrazhenskaya. 11. The Use of Paper-Chromatographic Methods in Classifying Actinomycetes, by A. I. Korenyako, N. S. Kirillova, and N. I. Nikitina. 12. The significance of Immunobiological reactions in identifying Actinomycetes, by N. K. Solov'eva and I. D. Denova.

From the list of the papers presented it is apparent that all the basic problems of classifying actinomycetes found expression in the program of the meeting.

To all of the participants of the conference it was evident that biological species is indeed an objective reality, but also that the properties of each species are characterized by a complex of determinants depending solely on their own pecularities and properties or characteristics.

The matters of controversy concerned the significance which different investigators assign to various determinant characteristics and the evaluation of the stability of one or another characteristic.

## The Significance of Morphological Characteristics

N. A. Krasil'nikov considers that several morphological characteristics have a leading role in the nomenclature of actinomycetes, in particular the organs of reproduction, a characteristic which is hereditarily determined, stable, and easily observed. However, in his opinion the sporangia, the manner of spore formation, and spore shape, although appearing to be stable properties, cannot serve as the basis for differentiating actinomycetes into species since different species of actinomycetes are encountered in a culture with identical spore and sporangial structure. But for determining the strain of a species the form of the sporangia and the manner of spore formation are quite important characteristics for each determined species of actinomycetes. T. P. Preobrazhenskaya, E. S. Kudrina, M. A. Sveshnikova and T. S. Maksimova consider that primary significance cannot always be assigned to the structure of the sporangia as a species characteristic. They feel this characteristic is less constant than, for example, the color of the air and substrate mycelia. Their observations of cultures with spiral sporangia indicate strains with straight sporangia are obtained on transference. This characteristic is preserved upon further transference. These authors noted that, among the actinomycetes, so-called conjugated species are encountered as well as other variants differing among themselves only in the structure of the sporangia. Furthermore, A. I. Korenyako, N. I. Nikitina and E. I. Andreyuk, Yuan Tsi-sheng, S. M. Rudaya, and others demonstrated that the form of the sporangia and the manner of spore formation are more stable characteristics than cultural and physiological characteristics.

Undoubtedly, several morphological characteristics, particularly the ability to form certain reproductive organs, are stable and hereditarily established characteristics during the long evolution of these organisms and are basic in differentiating actinomycetes. These can be used for differentiation into a higher nosological categorization (genus, order, and family).

However, not all the peculiarities of reproduction have the same classification value. For example, such characteristics as the structural form of the sporangia (the presence of spirals and their character or the absence of spirals) and the form of the spores, despite the comparative stability of these features in growing the organisms under set conditions, cannot be used to differentiate actinomycetes into groups or into species within a genus.

The aforementioned characteristics can be used to characterize cultures of a known species.

From this it follows that the structure of the sporangia and the forms of the spores, as with the majority of other characteristics, depends on the content of the medium and the conditions of growth. Therefore the absence of spiral sporangia during growth on several media does not always signify the advent of a stable variant with new morphological characteristics. It is important to know what conditions are more favorable for the appearance in the given culture of these peculiar forms of reproduction. It is doubtful if it is expedient to isolate cultures which are similar in their antibiotic production and in other properties if they differ only in the form of their sporangia. The authors ascribe these things to variability (Actinomyces violaceus and A. violaceus rectus, etc.).

As to such morphological characteristics as the thickness of mycelia and the nature of their branchings, they unquestionably have no nosologic significance.

The electron microscope has established three types of actinomycete spores—smooth, spiny, and hairy. There are possible transitional forms.

A problem arises in using this characteristic for classifying actinomycetes. Some investigators consider this characteristic important, others as an auxiliary characteristic. T. S. Maksimova, T. P. Preobrazhenskaya, E. S. Kudrina, and M. A. Sveshnikova reported their results of studying spores of 800 strains belonging to 122 species. The author thought that they found a correlation between the structure of the spores and the color of the air mycelia. The character of the sporangial structure is a rather constant characteristic. The authors are not in agreement as to the diagnostic value of the structure of the sporangia for different species; for some it is an important sign, for others an insignificant one.

Undoubtedly data on the structure of the sporangia should be included in characterizing species that have been studied adequately. It would be most desirable to have further investigations in this area.

The data presented by A. A. Prokof'eva-Bel'govskaya and Z. V. shamina were of special interest.

The authors carried on detailed cytological investigations on the structure and growth of colonies on Czapek's medium. Representatives from four different species of Actinomyces were used—A. streptomycini, A. griseus, A. longisporus, and A. violaceus. It appeared that the type of development and the microscopic structure of the substrate portion of the colony differed in all species studied and was highly specific for each. The authors felt that the data on the microscopic morphology of colonies could serve as an important additional morphological characteristic and could be used successfully for classifying species. They felt these characteristics could prove valuable for arranging a natural classification of actinomycetes.

The meeting recognized the importance of more widespread investigation in the areas of species specificity, type of development, and microscopic structure of actinomycete colonies and noted the necessity for organizing studies of cytochemical and biochemical properties of actinomycetes for use in a classification system.

## The Significance of Cultural Characteristics

Different points of view were expressed as to significance of the color of the air and substrate mycelia for classification of actinomycetes. These characteristics are considered by a number of investigators as being basic diagnostic characteristics (Baldacci, Gauze, etc.)

T. P. Preobrazhenskaya, E. S. Kudrina, M. A. Sveshnikova, and T. S. Maksimova consider the color of the air and substrate mycelia to be rather stable charac-

teristics; these were maintained in the cultures investigated by them during the course of 4-5 years of observation and growth on favorable media. They found that changes in the color of the air mycelium were connected with poor growth or with renewed growth in the cultures. N. A. Krasil'nikov, N. I. Nikitina, and others noted that the color of the air mycelium is not a constant characteristic and varies widely, depending on the content of the media and the conditions of cultivation. A more stable and heriditarily transmitted characteristic is the color of the actinomycete culture, which depends on pigment production. In such an instance the color has a fortuitous character and can differ in various cultures. However, the pigment is variable and its character depends on the conditions of growth, the content of the medium, etc. In the opinion of N. A. Krasil'nikov, the color of a culture and the air mycelium as a morphological characteristic has group significance but not species significance and thus cultures of actinomycetes of certain groups or sections can be linked according to this characteristic.

G. F. Gauze in his comments also noted color of air mycelia as a group characteristic so that at times closely related species are encountered which differ among themselves in the color of the air mycelia.

The results of an international experiment to determine the significance of different characteristics in the nomenclature of actinomycetes was of great interest at the conference. In this experiment a number of countries of Europe, the Americas, and Asia took part. Twenty-six cultures of actinomycetes of different species were studied according to a unified scheme and methodology using media of uniform content. The Institute of Microbiology of the Academy of Sciences of the USSR and the Institute for the Investigation of New Antibiotics of the AN SSSR took part in this study. The results of the international experiment established that the most persistent properties of the actinomycetes were structure of the sporangia and formation of the melanin pigment.

The structure of the sporangia and the color of the air mycelium was relegated, consequently, to a secondary place. The color of the substrate mycelium turned out to be the most variable characteristic.

It must be recognized that the color of the air and substrate mycelia are characteristics which are treated in many investigations as being of primary importance and to which, in fact, the majority of investigators assign primary importance in classifying new cultures according to groups and subgroups.

## The Significance of Physiological Characteristics

All the participants in the conference were agreed that the usual physiological characteristics which are widely used in differentiating bacteria, such as the ability to liquefy gelatin, curdle milk, reduce nitrates, invert sugar, and hydrolyze starch, do not have any real significance in classifying actinomycetes. Some of these characteristics are too widely spread among the actinomycetes and are peculiar to the majority of them, while others change rapidly with time and cannot be correlated in classifying cultures. Therefore these characteristics are useful only for characterizing a certain strain.

N. A. Krasil'nikov and N. I. Nikitina showed that the ability to utilize certain carbohydrate compounds can be used successfully in differentiating species within certain narrow groupings.

## Antimicrobial Properties and Specificity of Antagonisms

One of the most hotly debated problems of the conference was that the specificity of antagonisms, i.e., the interrelation of actinomycetes dependent on the antibiotics produced by them. N. A. Krasil'nikov surveyed the ability of cultures to form certain antibiotics as a species characteristic, on the basis of which differentiation into species could be made, using morphological and cultural properties as well.

This property, in his opinion, is a very stable characteristic and in a series of experiments he could not obtain from cultures producing a certain antibiotic any variant capable of producing another antibiotic or variants completely losing their ability to produce the antibiotic peculiar to them. However, he considered that the specificity of antagonisms must be considered together with other characteristics and should be used for differentiating cultures within a group established by morphological and cultural characteristics.

A. I. Korenyako and many other participants in the conference were of the same opinion.

As is well known in the literature, there are indications that a single antibiotic can be produced by cultures of different species and that this characteristic is not stable.

For example, Waksman considers that the ability of cultures to produce antibiotic compounds can hardly be considered a basic permanent feature for the characterization of species, inasmuch as this property is variable. In his opinion, this property can be used for describing variants.

T. P. Preobrazhenskaya, E. S. Kudrina, M. S. Sveshnikova, and T. S. Maksimova noted that actually, in the majority of instances, each antibiotic is formed by cultures of one species. However, they often observed that the same antibiotic is formed by cultures belonging to different species, or one species forms several antibiotics simultaneously. Therefore the ability to produce antibiotics is now considered not as a basic species characteristic but as an auxiliary one.

G. F. Gauze noted that instances of different species of actinomycetes producing the same antibiotic occurred, but were relatively infrequent. For example, in his laboratory it was noted that oxytetracycline is produced not only by A. rimosus but by a culture of A. aureofaciens.

In his opinion, different antibiotics are produced by different species. This characteristic, despite some exceptions, is quite valid and is indeed a basis for practical work.

N. A. Krasil'nikov, A. I. Korenyako, and G. F. Skryabin consider that the authors who maintain that the same antibiotics can be produced by cultures of different species are in error only as regards certain

species or in the exactness with which the nature of the antibiotics are established. As a matter of fact, instances in the literature show that streptomycin is produced by A. bikinensis and A. galbus as well as by A. streptomycini. According to the data of A. I. Korenyako, a comparative study of cultures of A. galbus showed that it differs from A. streptomycini not only in the form of the sporangia, color, and antagonism but also in its antimicrobial spectrum. Consequently these cultures form different complexes of antibiotics.

The participants in the conference gave much attention to the problem of the methodology by which antagonisms between cultures could be expressed.

N. K. Solov'eva noted that the method for determining such interrelationships is frequently of limited value because of two phenomena observed—inhibition, which is difficult to differentiate from true antagonism, and lysogenesis. It is necessary that authors who propose this method clearly note how it was used by them.

N. A. Krasil'nikov, O. I. Artamonova, and others consider that cultures of one species do not usually inhibit each other. However, there are instances of autoinhibition when cultures of one species do inhibit each other. Autoinhibition can be brought about with phage and also by certain compounds of the necrohormone type. These compounds are formed only on certain media and they can be isolated from the antibiotic. Therefore, in determining antagonism a medium must be used on which compounds of this type are not formed but antibiotics are.

M. A. Sveshnikova and others noted that widely distributed species are encountered which can be unified according to their antimicrobial spectrum but which differ in their antagonism so that cultures of one species inhibit the growth or another.

G. F. Gauze maintained the point of view that the specificity of antagonism is a peculiar manifestation of antimicrobial properties of a culture. This characteristic is important. However, there are several well-known instances of autoinhibition, and this problem needs further study. G. F. Gauze in particular feels it is necessary to investigate the phenomenon of autoinhibition.

It seemed to us that in deciding so important a problem as the significance and suitability of the use of the phenomenon of antagonism of actinomycetes in differentiating them, one must approach the problem bearing in mind the following: 1) It is evident that the vast majority of cultures of actinomycetes are resistant to the antibiotics which they produce. 2) The antimicrobial spectrum, including the effects on certain actinomycetes of each antibiotic, should be strictly specified and the antibiotic and its properties should be characterized. 3) The synthesis of antibiotics is connected with certain metabolic pecularities of any given culture. 4) The ability to produce certain antibiotics is one of the stable characteristics of the culture. 5) No one has yet shown convincingly that cultures of actinomycetes belonging to distinctly different species produce absolutely one and the same antibiotic or a single complex of antibiotics. This last question is so important that it would be worthwhile for several Institutes to conduct a joint study of these cultures of actinomycetes of different species that have been described as producing one and the same antibiotic. This would allow solution of this thorny problem on an absolutely objective basis.

The use of pure antibiotics in identifying cultures would undoubtedly eliminate a number of difficulties arising from the use of solid and liquid media in studying interrelationships of cultures. As is well known, in studying these phenomena the method of agar blocks is used, in which a block from test cultures previously grown on agar media is placed on the surface of the test culture.

Our own observations have indicated that in studying the antagonism of cultures of one species by this method valuable results are obtained.

For example, we observed that cultures of A. streptomycini are never able to inhibit each other, but possess the ability to inhibit and be inhibited by certain cultures of other species. In cultures of several other species, especially the pigmented forms, the phenomenon of autoinhibition is frequently observed. Apparently, compounds which cause autoinhibiton are of variable character and nature. According to our data and the data of É. S. Khavina and others, among the actinomycetes the phenomenon of lysogeny is widespread. It was first shown by us that the phenomenon of autoinhibiton among actinomycetes is connected with the lysogenic condition of the experimental culture and is caused by sensitivity of the surface culture to phage which are situated in the transferred culture. Inhibition caused by phage is rather easy to distinguish from that caused by other factors, but differentiation among the latter is more difficult.

It is well known that many bacteria are capable of producing particular compounds named bactericidins. Among these compounds there are some rather specific ones which do not inhibit their own cultures or inhibit them only in rare instances, e.g., colicidin. It has further been established that other cultures produce compounds which always inhibit their own cultures. Megacidin, which is produced by many strains of Bacillus megaterium, is related to this group of compounds.

Among the compounds produced by actinomycetes undoubtedly there are those which inhibit their own culture but also those which do not act on them. The study of these compounds and their influence on their own cultures has great significance.

It should be kept in mind that the antagonism between cultures observed during experiments on artificial solid or fluid media does not reflect true antagonisms of cultures and frequently the matter depends on the content of the medium, the conditions of growth, the age of the culture, and other factors.

Despite the fact that there are difficulties in using the proposed method of antagonism in identifying cultures of actinomycetes, many years of experience has shown that it is useful in facilitating work on the nomenclature of actinomycetes and sometimes can uncover differences which are not evident from other

methods of investigation. In our opinion these methods ought to be used.

However, one must keep in mind the negative aspects of this to avoid erroneous conclusions and in each instance carefully analyze the data obtained to determine the causes of autoinhibition.

The Variability of Actinomycetes

It is evident that a basis for classifying actinomycetes must be founded on the least variable characteristics. Therefore particular interest was centered on data as to the variability of actinomycetes observed under ordinary conditions of cultivation and also the effect on cultures of various exogenous factors.

S. I. Alikhanýan, L. P. Borisova, and L. I. Erokhina reported to the meeting the results of their investigation on the study of the influence of mutagenic factors (UV and x-ray) and of phage on A. streptomycini, A. aureofaciens, A. rimosus and A. olivaceus.

The mutants obtained under the influence of the indicated factors differed from the original cultures in one, two, or a number of characteristics. Instances were observed of complete loss of ability to produce antibiotics and acute changes in morphological properties, color, and forms of spores and their capsules, the antibacterial spectra and sensitivity to antibiotics and actinophage. The changes under the influence of mutagenic factors can involve any characteristic and each characteristic can be changed independent of another.

The report of S. I. Alikhanyan et al. also enlivened the discussion. N. A. Krasil'nikov, V. A. Tsyganov, and A. N. Korenyako expressed a number of objections, particularly as to the possibility of experimentally obtaining cultures with new antibiotics and with acutely changed morphological properties.

It seems to us that the data on experimental variability has great significance in the nomenclature of actinomycetes as it allows the stability and lability of one or another characteristic in certain cultures to be determined, as well as possible ways of bringing about new species.

However, we must differentiate changes occurring due to factors which frequently cause profound alterations in the hereditary property of the culture from changes observed under natural conditions and under laboratory conditions of growth.

Undoubtedly, under the effects of such potent factors, one can radically change many characteristics, but this does not decrease their value for classification. The matter which principally interests us is the problem of recognizing and classifying forms which are isolated from natural sources. The process of variability under natural conditions continues due to the influence of other factors, especially natural selection, which contributes to the stabilization of those characteristics having species significance.

The mutants obtained due to mutagenic factors, despite the acute changes in important characteristics, should be considered as radiation variants of the original strain and not new species forms. The report of S. I. Alikhanyan, L. N. Borisova, and L. I. Erokhina roused great interest when it suggested the use of hybridization phenomena, i.e., the ability of strains to breed as a fundamental biological characteristic making it possible to judge the genetic relations of cultures of different groups.

Thus, in their opinion, the mycelia of different cultures which are phylogenetically different do not merge (that is, heterokaryons do not emerge).

The attendees agreed that a study of variability is important in the nomenclature of actinomycetes in that it can enrich our knowledge of the relation between different species and give us some idea of the possible forms that each species might assume under known conditions. In this connection studies should be made on the variability in cultures which are being widely used at the present time and also representatives of important standard cultures.

The attendees felt that studies should be conducted on the possibility of varying important species and their properties (the ability to produce antibiotics, the structure of the sporangia, etc.).

Further investigation of the possible use of the method of hybridization in the problem of nomenclature was recognized as being essential.

Serodiagnosis of Actinomycetes

The report of N. D. Solov'eva and I. D. Delova was devoted to this problem. On the basis of the literature and of their own observations the authors arrived at some conclusion on the future use of this method to identify cultures or their physiological relationship. The desirability of applying the agar precipitin reaction was noted as one of the most definitive of contemporary immunobiological methods.

V. A. Kuznetsov, G. F. Gauze, V. P. Tul'chinskaya, and G. K. Skryabin underlined the importance of serological methods and brought to attention the fact that very much still needs to be done as regards obtaining highly active specific antigens and antisera. It is necessary to study the antigenic structure of actinomycete cells and their different components. In the opinion of V. P. Tul'chinskaya basic information must be obtained on the isolation of those antigens which can be used for the precipitin reaction.

The Use of Actinophage in the Systematization of Actinomycetes

Data on the application of actinophage in identifying actinomycetes and the difficulties arising from this method were set forth in the report of Ya. I. Rautenshtein. It was pointed out by him that the characteristic of sensitivity or resistance to specific actinophage can be successfully used in differentiating actinomycetes in some instances into species and in others into subspecies. It is important to underline that this characteristic is quite stable. The observations of several years' duration on actinomycetes have shown that the preservation of the organism on various media and under different conditions of growth does not change their relationship to specific actinophage.

Unfortunately, the number of specific actinophages is as yet rather limited. The majority of actinophages isolated from soil are polyphagic and not suitable for use in classification.

V. D. Kuznetsov and E. S. Khavina reported that several specific actinophages were successfully used by them in classifying actinomycetes of several species.

A resolution of the conference noted the importance of this problem and thus recognized the necessity for broadening investigation toward isolation and study of actinophage and testing their specificity. The conference also noted the necessity for creating a collection of standard actinophages at the Institute of Microbiology of the Academy of Sciences of the USSR. At the same time the conference recognized the importance of studying the phenomenon of lysogenisis among actinomycetes and the influence of symbiosis of actinophage on the biological properties of lysogenic cultures.

The Use of Methods of Chromatography

Recently the method of paper chromatography has been used more widely in differentiating actinomycetes.

A number of modifications of this method have been proposed. A. I. Korenyako, N. F. Kirillova, and N. I. Nikitina reported the result of their investigation of the possible use of paper chromatographic methods in differentiating actinomycetes. The authors came to the conclusion that paper chromatography is not well suited for establishing species differences in outwardly similar actinomycetes and that it has limited value in the matter of identifying species. Paper chromatography can be used only in combination with other methods. Chromatographic evidence should be obtained not on a single culture but on several. V. A. Tsyganov in his report underlined that he does not agree with the conclusion of A. I. Korenyako on the fact that chromatographic methods are of little use in elucidating the species differences. In his opinion, if the characteristic of forming certain antibiotics is of great diagnostic significance then one must also recognize chromatographic data. It is another matter that the proposed method is of little use for the indicated goal. Consequently it is necessary to perfect the method of chromatography.

A. V. Markovich noted that many actinomycetes form several antibiotics and that the antimicrobial spectrum in itself should be considered together with chromatographic data to evaluate its full diagnostic significance. This would allow one to find out how many antibiotics are produced by a single culture.

The Problem of Nutritive Media

A special report of E. S. Kudrina, T. P. Preobrazhenskaya, M. A. Sveshnikova, and T. S. Maksimova was devoted to the problem of the significance of the content of nutritive media in elucidating morphological and cultural characteristics of actinomycetes. This problem was touched upon in the report of N. A. Krasil'nikov and that of O. I. Bershova and V. A. Tsyganov. Many workers came to the unavoidable conclusion tthat the content of the medium has a great effect on the morphological and cultural as well as antimicrobial properties of the actinomycetes. Several investigators pointed out the great randomness in the selection of media in describing new species so that frequently non-standard and unobtainable media are used so that other investigators cannot do comparative studies on various cultures.

In the hope of straightening out this undesirable situation the conference set up a special subcommission made up of representatives of the Institute of Microbiology of the Academy of Sciences of the USSR, The Institute for Investigation of New Antibiotics of the Academy of Medical Sciences of the USSR and The Institute of Antibiotics of the Ministry of Health of the USSR in order to draw up a list of media and their contents which would be recommended as obligatory in studying and describing cultures of actinomycetes and they instructed them that in setting up this list of media they follow the recommendations of the International Handbook as well as the media proposed by the authors at this conference.

It was recommended to this commision that a scheme be established for describing the cultural and morphological and other properties of actinomycetes with an indication as to which media should be used in eliciting each characteristic.

The list of media recommended by the subcommission and the scheme for describing cultures should be published during 1961 after approval by the specially created commission.

The conference called the attention of the investigators to the fact that in describing new forms or in reestablishing old species and in assigning names to them one must strictly conform to the International Codex of botanical and microbiological nomenclature.

Furthermore, the conference considered it quite important that in describing new species references be made to previous literature on sources and also data of comparative studies of the described culture with closely related standard cultures and that differential diagnostic features be given.

In this regard it was decided that the presence of standard cultures would significantly facilitate the work on the nomenclature of newly isolated cultures; therefore the conference created a special commission to examine the cultures of actinomycetes which are known to exist in different stocks and to isolate from them those which might be recommended as standards. The isolated standard cultures could be preserved in the following institutes: The Institute of Microbiology of the Academy of Sciences of the USSR, The Institute for Investigation of New Antibiotics of the Academy of Medical Sciences of the USSR, The All-Union Scientific Research Institute of Antibiotics (Moscow), The Leningrad Institute of Antibiotics. The staff of the commission included representatives of these same institutes.

The conference commissioned this group to isolate for detailed study those cultures whose species specificity is doubtful and also to solve other disputed issues important in nomenclature of actinomycetes.

The discussion which has been carried on by the journal "Mikrobiologiya" on the problem of classifying actinomycetes and also the conference devoted to this problem undoubtedly have had positive implications.

They, on the one hand, have contributed toward bringing together the vast work of the last few years on this problem by different scientists in the USSR and abroad.

The broad discussion of the accumulated factual material and different interpretations of it permit, on the other hand, better and more complete understanding of the various points of view in the nomenclature of actinomycetes in order to establish a rapprochement on these problems from different points of view as well as on those problems which remain debatable and which require a practical solution.

The conference made note of a series of practical investigative and organizational methods directed toward facilitating a more successful solution of the problem of classifying actinomycetes.

The conference also underlined the importance of close contact between investigators of different institutions working on actinomycetes and the necessity for solving the matter in dispute by cooperative experiments, exchange of culture media, and simultaneous examination of materials.

An important achievement of the conference was the organization of the special commission and the subcommission charged with working out a list of recommended media and a scheme for describing morphological and cultural properties as well as for developing a list of standard cultures.

Upon completion of the task of the commission and the subcommission the published results will undoubtedly contribute to a successful development in our country of investigations concerned with the study of actinomycetes and their classification.

# ALL-UNION CONFERENCE IN NOVOSIBIRSK ON MICROBIOLOGICAL METHODS OF COMBATTING HARMFUL INSECTS AND RODENTS

I. N. Gritsenko

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From March 30 to April 1, 1960, a conference was held in Novosibirsk on microbiological methods of combatting agricultural and forestry pests and on the microbiology and pathology of insects.

The conference was organized by the Biological Institute of the Siberian Division of the Academy of Sciences, USSR. Eighty-five representatives of more than thirty scientific research establishments and institutions of higher learning in Siberia and the European part of the USSR participated in it.

The conference heard and discussed 39 reports: 5 on general questions; 5 on directed variability in microorganisms; 4 on the diagnosis of insect and rodent diseases; 13 on the preparation and application of microbial preparations; and 12 on combatting the diseases of beneficial insects.

In a paper, "The theoretical principles of the microbiological method of combatting agricultural and forestry pests," V. I. Poltev pointed out a number of advantages of the microbiological method in comparison with other methods of exterminating harmful insects and rodents. With the aid of microbes which had undergone directed alteration, it is possible to produce epizootics among only certain species of pests and to bring to harm to the beneficial fauna.

However, the onset and development of an epizootic is influenced by the resistance of the pest, its numbers per unit area, the virulence and dosage of the microbial preparation, the resistance of the microbe to the effect of environmental factors, the method of spread of the infection, and the development of acquired immunity. It is expedient to have two or several microbial preparations against a single species of pest; moreover, the preparations must be applied in combination with baits which are eaten readily by the respective species of pests. The microbial preparations must not injure the beneficial fauna.

M. I. Prokhorov, in his report, characterized the stages of development of the microbiological method of rat extermination and its great possibilities. At the present time, Merezhkovskii's, Isachenko's, and Danich's bacteria and bacterium No. 5170 obtained by them are being successfully employed in our country for the extermination of rodents.

A. I. Sidorenko reported on the presence in the soil of microbes which inhibit the activity of certain insects which are pests of agricultural cultures. The author succeeded in infecting adult beet weevils with a culture of a nonpigmented variant of Bacterium prodigiosum.

S. S. Folitarek presented extensive data on the distribution and economic damage inflicted by the water rat, while Kolomiets reported on damage brought about by the Siberian silkworm.

D. F. Petrov reported on the possibility of selecting needed forms of bacteria by means of using inositol, thiamine, adenine, etc. as selective factors. The author worked out the penicillin method of microbial selection.

In order to increase the virulence of entomopathogenic fungi, A. A. Evlakhova employed physical and chemical agents: DDT, HCCH (hexachlorocyclohexane), ultrasound, UV, x-rays and gamma rays. The fungal variants obtained as the result of the treatments were selected according to cultural characteristics. The virulence of the selected variants was tested on the harmful eurygaster and the lesser apple worm. The author notes that the utilization of strong-acting factors is promising with regard to increasing the virulence of entomopathogenic fungi.

L. M. Tarasevich and E. F. Ulanova, in a paper, "A contribution to the mechanism of the resistance of polyhedrons," showed the resistance of the latter to various external treatments and dependence on the disulfide groups of cystine. The latter is broken down by alkalis and acids with the formation of lacthionine.

A. B. Gukasyan reported on the positive effect of a strain of Bacillus dendrolimus which he isolated on the caterpillars of the Siberian silkworm. The young and the oldest caterpillars proved to be particularly sensitive to the preparation.

O. I. Shvetsova reported that, in spore-forming entomopathogenic bacteria, the formation of crystalline inclusions has been noted along with spore formation. The taxonomic significance of these inclusions has not yet been sufficiently substantiated, but this characteristic should be one of the most important in the selection of pathogenic strains. In selecting virulent strains of entomopathogenic spore-forming cultures the formation of inclusions, the ability to affect various groups of insects, and antigenic properties must be taken into consideration.

I. N. Gritsenko reported on the isolation of pathogenic microorganisms from the water rat. Certain strains of the paratyphoid and erysipeloid group were found to be suitable for directed increase in virulence. Microbial preparations were obtained which, when used to infect water rats, guinea pigs, rabbits, and pigeons orally, caused disease and death only in water rats.

A. E. Karpov presented data on the wide occurrence of transmission of latent nuclear polyhedral disease

virus among insects. This can be transmitted hereditarily through eggs, remaining inactive during a number of generations; however, under the influence of suitable ecological conditions, it is transformed to the active state, causing polyhedral disease and death of the insect. The author established that, of the treatments tested by him, the most effective for causing polyhedral disease in the silkworm is mild irradiation of caterpillars with doses of 5-6 thousand roentgens. Experiments showed that the appearance of polyhedral disease also depends on the developmental phase of the mulberry silkworm.

G. M. Rybakova used the agglutination reaction to identify erysipeloids, while Trilenko used it to identify the agent of American damp rot.

V. A. Trilenko reported that strains of the agent of American damp rot with altered O-antigen exist in nature. These data make it possible to approach the study of humoral and cellular immunity in the American damp rot of bees more correctly.

In their papers, A. A. Evlakhova, I. S. Velitskaya, Ē. R. Zurabova, M. G. Gandman, L. Ya. Sintsova, O. S. Il'in, I. N. Gritsenko, R. A. Ryabkova and others suggested the most favorable media and conditions for growing virulent microbes against agricultural and forestry pests.

A. E. Khrutskii presented comparative data on the effectiveness of Merezhkovskii's and Isachenko's bacteria and No. 5170 against the suslik. The author established that adult susliks are more susceptible to No. 5170 bacteria. The lethal dose for them when infected orally is 8 billion microbial cells per 1 ml. Death of the susliks reached 90–100%. Young susliks are more susceptible to infection and death from Merezhkovskii's bacteria. The data of spring-fall field experiments showed that the bacterial preparations were more effective in the spring, when death of the susliks reached more than 90%, while in the fall period, only 40–44.3% of them died. The author explains this by the fact that, in the spring, the resistance of susliks decreases sharply following hibernation.

E. V. Orlovskaya reported data on the death of the period butterfly, the brown-tail moth, the tent caterpillar, and the gypsy moth from polyhedria. In the gypsy moth, polyhedria frequently coincided with nosematosis, in the tent caterpillar – with entomofluorosis, and in the brown-tail moth – with nosematosis and entomofluorosis. The author points out that the death of the period butterfly and tent caterpillar from polyhedria is associated with temperature conditions in the spring period, rather than with the developmental phases of the insect. In artifically created epizootics, it is necessary to introduce the agent into the habitat of the pest before the onset of its mass reproduction.

T. A. Shekhurina characterized the conditions determining the effectiveness of entomopathogenic fungi with respect to the harmful eurygaster.

A. Ya. Leskova reported on the high efficiency of entobacterin-3 against the apple tree moth, the cabbage butterfly, and the cabbage moth.

Several reports were devoted to questions of the occurrence and efforts to combat the diseases of useful insects.

The participants of the conference adopted a resolution in which they noted that an urgent problem of working out effective methods and measure for combatting agricultural and forestry pests and for the protection of useful insects confronts scientific research establishments and technical workers. Along with the development of chemical preventative measures, it is necessary to improve existing, and to develop new microbial preparations of sporogenous and nonsporogenous bacteria, viruses, rickettsiae, molds, and algae.

In the resolution, the most important scientific and scientific-organizational measures are enumerated, the accomplishment of which is essential for the more effective utilization of microbiological methods in combatting agricultural and forestry pests.

# THE STATE OF ALGOLOGY IN THE HUNGARIAN PEOPLE'S REPUBLIC

S. V. Goryunova and L. K. Osnitskaya

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We had the opportunity to become acquainted with the achievements of Hungarian scientists in the field of algology at the time of a scientific assignment to Hungary in February-March of 1960.

It should be noted that considerable attention is accorded to algae in the Hungarian People's Republic. Thus, the heads of three departments of botany (institutes, in Hungarian) — Prof. T. Khortobadi (University of Agriculture in Gedel), Prof. G. Ukherkovich (University of Humanities in Szeged), and Prof. I. Kish (Pedagogical Institute in Szeged) — are algologists. Candidate of Sciences G. Semesh is working at the Botanical Gardens attached to the University of Budapest; É. Kol — a worker at the Museum of Natural History associated with the Ministry of Agriculture in Budapest — is now retired, but continues to visit her laboratory and to maintain cultures of algae. P. Polek works at the Department of Microbiology at the University of Humanities in Budapest. Five scientific associates — Candidate of Sciences O. Shebesh'en; Candidate of Sciences L. Felfoldi; Candidate of Sciences B. Yanko; and Fellows Zh. Kalko and G. Tamash — are engaged in the study of algae at the Biological Institute of the Hungarian Academy of Sciences.

The majority of the scientists enumerated, i.e., Prof. Khortobadi, Prof. Ukherkovich, Prof. Kish, Candidates of Sciences Semesh, Kol, Tamash, and others, are taxonomists; Candidate of Sciences Felfoldi and Fellow Kalko are physiologists, and Candidate of Sciences Yanko is a geneticist.

The investigations of Prof. Khortobadi are devoted to the determination of interrelationships between algae and fish productivity of bodies of water.

He feels that the detailed study of animals and plants in fish ponds and the elucidation of the character of their interrelationships with the environment are an essential requirement for modern fish husbandry. In his opinion, without knowing the members of biocoenoses and the qualitative and quantitative relationships among the, it is impossible to obtain a clear picture of the life of the pond, without which, in turn, informed alteration of biosynthesis, i.e., the creation of possibilities for the systematic feeding of fish, is impossible. Prof. Khortobadi conducts observations on algae in artificial ponds at various fisheries in Hungary in collaboration with chemists, hydrologists, and other investigators. It has been established that, when appropriate measure are taken (melioration, addition of fertilizers, etc.), the ponds which are poorest in vegetation can be more fish-productive, while fish-productive ones can reach high productivity. Aside from the composition of the phytoplankton, natural fish productivity is also greatly influenced by the character of the soil, the transparency of the water mass, methods of artificial propagation of zooplankton, the addition of fertilizers, and the fish-feeding processes themselves. As the result of the systematic biological analysis of fish-culture ponds, it proved possible to make timely prognoses of blooming of the water and to undertake preventive measures for combatting it.

Among general questions of classification, Prof. Khortobadi's work on the study of the morphological characteristics of the structure of Scenedesmus and other algae should be noted. Among the forms of green protococcoid algae isolated by Prof. Khortobadi, some had high reproductive activity and were sent by him to the Biological Institute in Tikhani to be tested for their suitability for mass cultures.

Prof. Khortobadi made unusual observations on regeneration during the reproduction of Lyngbia, as well as physiological observations on the appearance of gas vacuoles in blue-green algae. The presence or absence of gas vacuoles can not be regarded as distinguishing characteristics in systematics, because in his opinion, gas cavities are the result of intensive metabolism and are characteristic for older cells.

The principal orientation of Prof. Ukherkovich's work is the study of algae from the point of view of ecology, coenology, and taxonomy.

He is conducting investigations on algal biocoenoses in the Tisza River, as well as in the small lakes remaining after the spring flood. A system of plant communities is being worked out, changes in which would be evidence of a change in the relative content of dead organic material of the Tisza River, a very urgent question connected with the discharge of industrial sewage and other contaminating factors. At the present time, he has set himself the task of making fixed observations, in conjunction with Soviet and Yugoslavian scientists, on the algae of the Tisza River along its entire length. At the present time, Prof. Ukherkovich is preparing for publication a monograph on the systematics of some species of the protococcaceae.

Prof. Kish is engaged in ascertaining the conditions for the development of so-called water-blooms, their types and history. He groups the types of "blooming" of water according to the period of time required for their development, by their duration in time, by their spatial distribution, and by quantity. He believes that for the water to start "blooming," not only external conditions are required (i.e., the presence of nutrients, light, appropriate temperatures, and other factors), but also the internal state of the bloom-causing

organisms themselves, i.e., coordination in the processes of development and reproduction. In the cycle of this series of work, Prof. Kish's investigations on the elucidation of the role of meteolological factors in the life of algae are unique. He established, for example, that the multiplication of certain algae occurs not only slowly and gradually, but sometimes suddenly as well. According to his observations, this suddenness can be caused by the equally sudden increase in the vital activity of the organism which, in turn, is explained by cyclonic and frontal changes in the weather. The rapid appearance of very great amounts of algae, according to his data, always marks the start of a cyclonic period. For example, when there is a very great number of species of Chlamidomonas incerta, two phases of development – the vegetative and reproductive – can be clearly distinguished. In the prefrontal period, i.e., 12-36 hours prior to the passage of a front, intensive photosynthesis and then spore formation occur in organisms which have accumulated in deep water. At the end of the cyclonic period, and in the majority of cases in the postfrontal period, the formation of gametes and copulation are characteristic, i.e., aside from internal factors, reproductive processes in algae are also determined by the environment, including the weather. The reason for conducting these works was an ancient Hungarian legend that the sudden blooming of water means the approach of rainy weather. Prof. Kish finds the cause for the connection between the start of "blooming" with the onset of rainy weather to be in the great sensitivity of algae to atmospheric changes, analogous to certain higher animals, as well as in an influence of a cosmic order manifested in the disturbance of frontal changes of the weather.

Prof. Kish is also engaged in studying the reproductive processes of Kirchneriella and other algae. He observed that, under unfavorable conditions, the cells of this organism broke up into small green bodies, i.e., into so-called hyperfragments measuring 1-2 $\mu$, which retained their viability and could develop into cells. He compares the formation and regeneration of hyperfragments to noncellular forms of bacteria. Candidate of Sciences Semesh's subject is connected with the study of the algae in the Danube. He is determining the seasonal aspects of the distribution of various species of algae, the connection between their development and the level of the water in the Danube, and other questions. At the present time, he is preparing a determinative key of diatoms for publication.

Kol is engaged in the study of psychrophilic algae. In her collection, there are forms which were isolated from the snows and ice of glaciers in Greenland, Iceland, and Norway.

One of the interesting works of Piroshko Polek is her isolation of nitrogen-fixing diatoms. This is the first mention of this fact in the literature.

Grets conducted investigations of the composition of bacteria and algae in the waters of a deep (1317 m) hot mineral spring (+75.8°) in the village of Chokanya Vishonta.

Shuba is engaged in studying the algal flora of caverns.

Very interesting work is being carried out by the algologists in the Biological Institute in Tikhani, since it is here that the husband-wife team of L. Felfoldi and Zh. Kalko are working on the isolation from nature and the bacteriological purification of cultures of green protococcoid algae. The purification of algae is accomplished by means of antibiotics, irradiation by ultraviolet, and plating. This work has already been in progress here for some years, and at present the laboratory already has over 100 different strains of bacteriologically pure cultures of green protococcoid algae. Strains having industrial significance have been selected, and the construction of equipment for mass cultivation will be undertaken in the very near future. The chemist E. Sabo has become interested in this work. A particularly favorable condition for the production of mass cultures of green protococcoid algae in Hungary is the abundance of thermal carbonate springs which can be successfully utilized for industrial installation, considerably reducing the cost of their exploitation.

Candidate of Sciences Yanko is about to undertake work on algal genetics, particularly the production of more active strains of green protoccoid algae.

Candidate of Sciences Shebesht'yan is engaged in studying the patterns of numerical relationships between phyto- and zooplankton in Lake Balaton. In determining biomass, she uses special molds which she prepares from plastiline according to Loman's method to determine volumes of the individual species.

Tamash is conducting systematic observations on phytoplankton in Lake Balaton. Working on the same subject as Shebesht'en and Gellert, she studied questions of the interaction between algae, etc.

In this review, we naturally cannot enumerate the entire range of questions with which Hungarian algologists are occupied, and seek only to give some idea of the principal trends and achievements in this field of the scientific collective of the Hungarian People's Republic.

# THE SIXTIETH ANNUAL MEETING OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

E. K. Afrikyan

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The 60th Annual Meeting of the Society of American Bacteriologists was held in Philadelphia, Pennsylvania, May 1-5, 1960.

Judging from the number of participants, the quantity of papers, and the amount of organizational work, this has been a more representative meeting in the history of the Society, and, according to the officers of the Society, was of greater significance than the International Congresses of Microbiology. There were 65 scientific sessions and five symposia, in the course of which there were presented about 600 papers. About 4,000 persons, representing all areas of microbiology and related sciences, attended the meeting. Among the participants were many prominent scientists from England, Canada, Hungary, Latin America and other countries. A large exhibit of varied items of supplies and equipment used in microbiology and manufactured by American firms was open during the meeting.

Professor A. A. Sinitskii (Leningrad) and E. K. Afrikyan, Chief of the Microbiology Section of the Academy of Sciences of the Armenian SSR, participated in the meeting. The latter read a paper "The causal agents of bacterial diseases of silkworm and use of antibiotics in their control," which proved to be of general interest.

American microbiologists demonstrate an active interest in the papers of Soviet Scientists. Soviet journals and books are being translated or are abstracted; many American scientists are studying Russian and becoming familiar with Soviet publications in their original form.

The organization of the 60th Annual Meeting of the Society of American Bacteriologists was well planned and carried out. Dr. M. W. Chase of the Rockefeller Institute was the chairman of the program committee. The festive opening of the annual meeting took place on May 1 when Milton R. J. Salton, a British scientist from the University of Manchester, lectured on "The Anatomy of the Bacterial Surface." The members of the American Academy of Microbiology also met on April 30 and discussed "Educational and Professional Standards in Microbiology."

The Society of American Bacteriologists is one of the oldest and largest scientific organizations in the United States. It was founded in 1899 and the roster of the Society currently lists 6200 members. Members of the Society include scientists working in the areas of general, industrial, medical, and agricultural microbiology. The Society has 31 local chapters in different states and in larger universities. National meetings of the Society are held annually. The Society publishes several journals, such as Journal of Bacteriology, Applied Microbiology, and Bacteriological Reviews. Members of the Society pay dues; the participants of the annual meetings must also pay a registration fee. The Society receives considerable financial aid from supporting memberships of industrial companies and firms. It must be pointed out that the governing body of the Society of American Bacteriologists allocates funds for scientific awards and prizes to individual scientists and laboratories for outstanding research.

The president of the Society has considerable power and is elected annually. Well-known American microbiologists have been elected to this office: Theobald Smith, Buchanan, Prescott, Navy, Waksman, Van Niel, Lancefield, and others. The current president of the Society is Charles Evans, an immunologist working at present in the area of epidemiology and immunology of poliomyelitis. H. R. Cox, one of the best known immunologists and a director of a very large laboratory of Lederle & Co. in New York, is the vice-president. The next, 61st, annual meeting of the Society of American Bacteriologists will be held in Chicago, April 23-27, 1961. A symposium on marine microbiology is scheduled to be held in Chicago at about the same time (April 20-22, 1961).

The program of the 60th meeting of the Society was grouped into five basic divisions of microbiology: industrial and agricultural, general, medical, immunology and virology, and physiology of microorganisms.

As indicated above, several symposia and a series of discussions were held.

Symposium. Selected Topics in Microbial Ecology. Considerable attention was paid to conditions and methods for isolation and cultivation of protozoa. Emphasis has been placed on the importance of ecological types of microorganisms and their significance in exploring the development of effective methods for combating insects microbiologically.

Symposium. Mechanisms of Bactericidal Action. Antibiotics as inhibitors of cell wall synthesis. Existence of bactericidal and lytic activities, primarily related to enzymes active in cell wall synthesis, has been demonstrated using as examples the results of studies with penicillins, cycloserine and certain other antibiotics. Particular attention was given to megacin and streptomycin.

Symposium. Control of Bacterial Metabolism. This problem was considered in papers from the point of

view of interaction of different enzyme systems and conditions for their synthesis.

Symposium. Complement. A definite role of complement in passive skin anaphylaxis was demonstrated. Two immunoelectrophoretically distinct globulins were isolated from human serum using the method of electrophoresis and chromatography on ion exchange cellulose. It has been established by means of ion-exchange chromatography that the third component of complement consists of two different compounds.

Symposium. Viral Agents in Relation to Tumors. Particular attention was given to the method of tissue culture. It was demonstrated that a series of transplantable tumors of viral etiology have been contaminated with other viruses. A review was presented giving evidence for the viral induction of leukemia in laboratory animals. Mouse leukemia virus was obtained from connective tissue of mice (Sarcoma 37). Using thin sections, electron microscopic studies of newly formed virus showed that the virus infection inactivates mitochondria, giving rise to inclusion bodies or viroplasts, which represent foci of virus synthesis. Inclusion bodies are rich in adenosine triphosphatase (J. W. Beard).

Round Table Session. Lysozyme as Related to Problems in Microbiology. Properties of lysozyme and its substrates, mechanisms of bacteriolysis, lysozyme and the host. It must be pointed out that in discussing the question of lysozyme, American bacteriologists pay primary attention to its practical application.

During the session devoted to analytical microbiology most of the reports were made by members of commercial firms and were devoted to the basic questions of application of antibiotics, vitamins, and disinfectants.

Reports presented during the 60th meeting of the Society of American Bacteriologists were most diversified in nature. This turned out to be, in part, an inadequate aspect of the program; however, after learning the contents of the papers, one can formulate an adequate picture of the current aspects of microbiological investigations in the United States.

A characteristic of microbiological studies in the United States is exemplified by strong emphasis on organic chemistry and biochemistry and utilization of the most recent methods in research. This direction in research allows close-range study of the mechanisms of investigated phenomena and determination of the important details of many industrial and technological methods.

First of all, one must examine the reports devoted to active microbial metabolites. Studies in this area have a leading place in microbiological investigations in the United States. The problems pertaining to the isolation of new antibiotics were hardly considered during the 60th meeting. However, these studies are receiving wider attention in the United States, particularly investigations of new and active antiviral anticarcinogenic antibiotics. The antibiotic phaeocystin was obtained from Antarctic algae similar to Phaeocystis porichetii (J. McN. Sieburth). Two to five grams of active ingredient was obtained from one kilo of algae. Phaeocystin is active against Staphylococcus aureus, Pasteurella mutocida, and Mycobacterium smegmatis. It is not toxic to white mice. Its chemical and pharmacological properties are being investigated.

A report was made about a study of antiprotozoal antibiotics (R. A. West, C. B. Murrell, P. W. Barbera). An agar overlay technique was used. The base layer, also serving as nutrient, was covered with ion-agar containing the test organism, such as Crithidia fasiculata, Ochromonas maehamensis, Tetrahymena pyriformis, Trichimonas foetus.

Several investigators (E. C. Herrmann, C. Engle, J. Gabliks, J. P. Rosselet, P. L. Perlman) reported on application of agar-diffusion method for investigating and determining antiviral agents. Chick embryo monolayer tissue cultures, grown in baking dishes, are infected with virus (vaccinia, Herpes simplex, West Nile, and Newcastle disease were used). After incubation of virus and cells for 2 hr the monolayer was overlaid by 2% agar containing lactalbumin hydrolyzate–yeast extract medium. Filter paper discs impregnated with tested solutions are laid on the hardened agar surface, and the dishes are specially sealed and incubated for 3-5 days for development of plaques. After this time, a second layer of agar containing indonitrotetrazolium chloride is added to permit visualization of virus-induced plaques. Up to 40 substances can be carried in a single dish. With the help of filter paper discs, antiviral activity may be detected with as little as 0.3 mg of the tested agent. Similarly prepared dishes can also be used for bioautography by applying an entire paper chromatogram to the agar surface.

S. F. Quan reported on the strong antibiotic activity of Kanamycin on Pasteurella pestis. Attention should be given to the high efficiency of combination of streptomycin and tetracyclin in experimental brucellosis, as well as to therapeutic effectiveness of amphoterramycin for candidomycosis.

A special session was devoted to the problem of microbial transformation of steroids. A group of investigators from Schering laboratories (G. Luedemann et al.), one of the leading commercial concerns for microbiological production of steriods, reported on studies dealing with transformation of steroids by different species of algae. The studies were limited to the order Chlorococcales; species of the following genera were investigated: Scenedesmus, Tetraedron and Chlorella. All species were grown under conditions of heterotrophic nutrition in pure cultures. The genus Scenedesmus proved to be most active and was used for more intensive studies. Cultures of this genus can remove the 21-acetate group by hydrocortisone and hydroxylate Reichstein's "Substance S," having polarity similar to that of hydrocortisone and 11-epihydrocortisone, has been established.

H. Murray and O. Sebek reported on dehydrogenation of steroids by Septomyxa affinis (strain ATCC 6737). Under aerobic conditions this culture degrades progesterone similarly to fungi, carrying out this process by the known mechanism of 1-dehydrogena-

tion. Progesterone is rapidly transformed into 1-dehydroprogesterone, 1-dehydrotestosterone, 1-dehydroandrostenedione, and $\triangle^1$-testololactone. Other hydrogenated steroids are catabolized in analogous manners.

Laskin, Thoma, and Trejo reported on the $\triangle^1$-dehydrogenating ability of microorganisms which convert cholesterol to cholestenone. The most active ability to form cholestenone has been established in bacterial cultures. A high degree of correlation exists between the ability of microbial cultures to convert cholesterol to cholestenone and to effect $\triangle^1$-dehydrogenation 3-keto-$\triangle^{1,4}$-pregnadiene.

Of interest are reports devoted to problems of microbiological formation of carotenoids. It is known that in the United States, many experiments are conducted on utilization of microorganisms synthesizing $\beta$-carotin as a source of provitamin A for animal nutrition. Possibilities for practical utilization of these studies are somewhate remote due to the unstable nature of provitamin under hydrogen. A study dealing with utilization of certain reducing agents for stabilization of carotenoids in dried mycelium of the fungus Blakeslea trispora was reported at the meeting. Santoquin, in a concentration of 0.25%, was the most effective of the tested substances.

Problems pertaining to studies of microbial metabolism occupied a prominent place in the program of the scientific sessions. Numerous presented papers were devoted to analysis of different processes of conversion of nucleic acids, purines, pyrimidines, amino acids, hydrocarbons, and inorganic compounds. Particular attention was given to microbial enzymes and elucidation of their mode of action. Similar investigations in bacterial physiology are carried out primarily in industrial microbiology in connection with technological studies.

At present, extensive studies are being undertaken in the United States on the development of means for utilization of microorganisms as sources of foods. In this connection particular attention is paid to amino acids and fats. Reports were made on biosynthesis of lysine by baker's yeast and neurospora and that of cysteine by Rhodospirillum rubrum as well as on the role of histidine, argenine, tryptophan, and certain other amino acids in physiology of different species of bacterial and molds. Frequent utilization of labeled atoms is made in studying problems of biosynthesis of amino acids and other compounds.

In the area of fats, reports were presented dealing with formation of ethylketones from fatty acids by Penicillium roquefortii, utilization of p-alkanes, and formation of lipids by a very active strain of soil proactinomycete, as well as the data about phospholipids in certain species of microorganisms. Reports about carbohydrates touched primarily upon metabolism of ribose and deoxyribose, different pentoses, and certain other compounds. Considerable attention was given to intensive studies of antigenic polysaccharides, their biosynthesis, and isolation in pure form.

The Canadian microbiologists Taber and Vining reported on studies of activity of certain strains of Claviceps purpurea in formation of alkaloids (ergo-alkaloids). Altogether 17 strains were studied, of which 13 formed alkaloids.

R. A. Rhodes and co-workers reported on formation of fumaric acid using a deep method in 20-liter fermentors. A culture of the fungus Rhizopus arrhizus NRRL 2582 was used. Fermentation was carried out at 33°C on a medium containing 10-16% sugar, sufficient amounts of $CaCO_3$ for neutralization of formed acid, and inorganic salts and ammonium sulfate as a source of nitrogen.

Taking into consideration that hair and epidermis are keratin compounds, a method for studying keratin activity was developed by Everett and Gordon. A medium consisting of minutely cut cleaned wool was used as a substrate. Enzymatically digested material is filtered after incubation and the absorption spectrum at 280 m$\mu$ is determined. The most active enzymatic agent determined in this manner, named "keratinase," was formed by A. fradiae.

Many reports dealing with classification of microorganisms were presented at the meeting. Particular attention was given to classification of mycobacteria and enteric bacteria. It must be pointed out that Americans widely utilize taxonomic determinations for many biochemical reactions and processes brought about by the differentiated cultures; morphological principles are not followed. Studies in systematics are carried out basically on stock cultures, frequently old and altered, without the necessary determinations of the ecologic nature of the species.

It was shown that rapid oxidizing reactions by cultures of Aeromonas and Pseudomonas can be a highly useful criterion for their differentiation from representatives of enterobacteria (B. Davis and W. Ewing).

The question of classification of ray fungi was discussed in several reports. It is apparent from the presented papers that on the basis of varied investigations American microbiologists are inclined to assume that cultures of different species of actinomycetes are differentiated by their cultural, morphological and physiological properties. Genera differing in these properties are placed into independent taxonomic categories.

Of interest are the data from electron-microscope studies of spore structure in the classification of actinomycetes. On the basis of spore structure there exist about 600 cultures of different species of actinomycetes. These are subdivided into four groups: those with smooth, warty (with growth), spiny (with spines, needles), or hairy spores. Surface structure is considered a constant and reliable criterion for classification of actinomycetes (H. Tresner, M. Davies, and E. Backus).

Papers read during the scientific sessions on soil microbiology dealt with different problems of biochemical transformation by soil microflora. L. Casida studied the ability of different groups of microorganisms to oxidize orthophosphites to orthophos-

phate: Of 23 cultures of yeasts, bacteria, and fungi only Pseudomonas fluorescens fixed orthophosphite and accumulated orthophosphates in the nutrient medium. W. Corcarelli and D. Pramer reported on isolation of a mold culture, identified as Arthrobotrys conoides, killing nematodes by means of networks of adhesive hyphal loops.

One session was devoted to phages; during different scientific sessions many papers on bacteriophagy were presented. Of interest is a report about obtaining new types of phages from insect-pathogenic cultures of Bacilius cereus, and studies dealing with phages for certain species of saprophytic mycobacteria.

During the meeting much attention was devoted to certain problems of bacterial spores and mechanisms of sporulation. Two scientific sessions were devoted to these problems. They were also discussed in the course of other sessions. It should be pointed out that along with many theoretical aspects of sporulation, papers of considerable practical importance were presented (spores of B. anthracis, their germination, virulence of B. anthracis, factors affecting spores and sporulation of Clostridium botulinum; problems of destruction of spore-bearing bacteria in food industry, etc.). Particular attention was paid to studies dealing with biochemical changes in sporulation and spore germination, activity of enzymes, chemical composition of spores, and especially to the dynamics of the content of dipicolinic acid.

A separate scientific session was devoted to marine microbiology (methods of microbial analysis, microflora of water reservoirs, and various independent problems). Casein hydrolyzate proved to be the best nutrient source for marine microorganisms (G. Jones). Of interest was the report on microbial corrosion of iron by marine sulfate-reducing bacteria (W. Blanton and C. Oppenheimer).

At the meeting devoted to food microbiology, reports were made on microflora of food products, the role of enterococci in food poisoning, and utilization of gamma rays and other agents in partial sterilization of food products.

Immunological studies predominate in the United States in the area of modern medical bacteriology and virology. In the United States different firms and commercial establishments (as for example, Lederle), specializing in commercial production of immune biologicals, established extensive scientific research laboratories, headed by important scientists.

Of the reports presented at a session in medical bacteriology, one must point out studies dealing with the use of fluorescent antibodies for early indications of pathogenic microorganisms and in diagnosis of diseases. This method is becoming firmly entrenched in medical practice. The following method is used for preparation of fluorescent antibodies: globulin fraction of immune serum is precipitated with ammonium sulfate, its nitrogen content is determined and it is coupled with flourescein isothiocyanate in definite proportion in albumin.

Reports were presented dealing with utilization of fluorescent immune bodies in identification of Actinomyces bovis, Brucella abortus, B. melitensis, hemolytic streptococci, toxigenic Corynebacterium cultures, and in diagnosis of whooping cough. The method used in application of fluorescent antibodies for identification of serotypes of a series of enteropathogenic cultures of enteric bacteria in children's diseases proved to be more sensitive and specific than other methods. As before, virologists in the United States pay primary attention to immunological investigations, which play a leading role. A marked interest is shown in rickettsiae. The problem of gaseous disinfectants (beta-propionlactone and others) and of aerosol methods of immunization are studied intensively. A series of reports was devoted to studies of infectious processes in animals exposed to radiation. Studies devoted to active microbial metabolites are in the forefront of microbiology in the United States: antibiotics (particularly anticancer and antiviral agents), hormones (particularly steroids, alkaloids, toxins, vitamins, and other actively produced compounds), microorganisms as sources of food products, organic, acids, enzymes, and other valuable compounds. In the same area must be considered studies for developing different immune agents, which are produced industrially in a well-organized manner.

# IN MEMORY OF E. V. RUNOV

**E. F. Berezova, V. P. Izrail'skii, A. A. Imshenetskii,**

**N. A. Krasil'nikov, E. N. Mishustin, A. N. Naumova,**

**and Ya. I. Rautenshtein**

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An important Soviet microbiologist, Efim Vasil'evich Runov, author of numerous works in general and agricultural microbiology, passed away on June 13, 1960, after a short illness.

E. V. Runov was born in 1901. On graduation from high school in 1918 he was drafted into the Red Army, and then was assigned to the K. A. Timiryazev Agricultural Academy to continue his education. He graduated from the Academy in 1924, having specialized, under the guidance of D. N. Pryanishnikov, in agronomy and soil management.

In his student days, E. V. Runov started to work as as assistant in the Narkomsem bacteriological-agricultural station. Subsequently he held the positions of investigator and head of a department. The bacteriological-agricultural station was reorganized and became affiliated with the All-Union Institute of Agricultural Microbiology, and E. V. Runov headed one of the departments of this organization.

Working at first under the direction of A. F. Voitkevich, and later independently, E. V. Runov carried out a series of valuable investigations in general and applied microbiology.

In 1930, while holding this position, E. V. Runov also began his teaching activity and for a long time was Assistant Professor in the Microbiology Department of the Institute of Large Meat and Dairy Cattle, and in the Institute of Production of Agricultural Machinery in Moscow.

From 1940 to 1950 E. V. Runov was the director of the Microbiological Laboratory of the All-Union Institute of the Cheese Industry. In 1950-1951 he worked as a scientist in the Institute of Animal Morphology, AN SSSR, and then moved to the Forest Institute, AN SSSR (at present, Forest Management Laboratory, AN SSSR) in the position of Director of the Microbiological Laboratory. He worked in this Institute for the rest of his life.

Of particular significance among Runov's general theoretical investigations were the studies devoted to cyclic transformations of nitrogenous compounds. In his early works he showed that certain saprophytic bacteria may form small amounts of nitrites in a medium; this was later confirmed by N. B. Nechaev.

His investigations in the area of denitrification are of particular interest. He showed that the amount of loss of free nitrogen in reduction of nitrates depends not only on anaerobiosis, but also on relation of nitrates to the nature of nitrogenous nutrition.

Paying considerable attention in his studies to milk-souring bacteria, E. V. Runov established a curious fact: it became evident that the fermenting complex transforming carbohydrates to lactic acid may function actively at temperatures above the maximum for development of the component bacteria.

One of Runov's earlier studies, carried out in collaboration with V. P. Izrail'skii (1924), is of particular interest. In it was shown that certain vitamins act quite favorably on multiplication of certain microorganisms.

For a very long period of time E. V. Runov was interested in dairy microbiology and was one of the best specialists in this area in the USSR. Together with A. F. Voitkevich he studied extensively the ripening of kumiss and of microorganisms having the principal role in production of this beverage. This allowed development of technological means of preparation of kumis in our resorts.

Substantial results were obtained by E. V. Runov in studying the ripening process of different cheeses and in utilization of starters, allowing not only a more rapid preparation of a given product, but also improvement of its quality. His observations of lysis of lactic acid bacteria by phage during manufacture of cheese, and on the biological significance of this process, are of considerable value and are imaginative in nature.

E. V. Runov carried out a series of investigations dealing with microflora of the gastrointestinal tract of farm animals and with improvement of digestion of rough fodder. He was well versed in this poorly studied area and always gladly shared his knowledge with microbiologists initiating studies of such problems.

E. V. Runov was always interested in soil microbiology. He studied partial sterilization of soils containing nonsymbiotic and symbiotic nitrogen-fixing organisms. Together with V. P. Izrail'skii and V. V. Bernard he published a monograph, "Bacteria in nodules and nitrogenation" (1932), which earned at that time a well-deserved reputation as the best Soviet book on symbiotic nitrogen-fixing organisms.

Towards the end of his life E. V. Runov worked primarily in the area of soil microbiology, devoting his time exclusively to studying the microbiology of forest soils of the Soviet Union. He carried out this work thoroughly. He utilized the wide network of stations of the Forest Institute, AN SSSR. E. V. Runov and his colleagues studied microbial association in differ-

ent soil regions of the USSR. These studies led to accumulation of valuable data demonstrating the strict specificity of microorganisms for each type of soil.

Taking an active part in investigations of forestation of steppes, E. V. Runov studied the influence of the forest growth on the microbial population of steppe soils, and clarified the interrelationships between grassy and forest vegetation. He was interested, in particular, in toxic products of microbial metabolism, to which he was inclined to assign considerable importance.

E. V. Runov and his laboratory made valuable observations on the development of mycorrhiza in woody plants growing in a steppe.

Runov's great learning and extensive experience influenced different associations of authors to enlist his services for preparation of study aids for universities, technical schools and courses. He was a co-author of a series of well-known text books.

Distinguishing characteristics of E. V. Runov were his modesty and extensive demands upon himself. He was awarded the title of Candidate of Biological Sciences by the K. A. Timiryazev Agricultural Academy for his work devoted to the ripening process of cheeses (1946).

In the memory of all who know E. V. Runov will always remain the charming image of this modest scientist, working honestly and fruitfully in his chosen area of science.

## DEFENSE OF DISSERTATIONS *

A. E. Kosmachev

Translated from Mikrobiologiya, Vol. 29, No. 5, pp. 786-790, September-October, 1960

### I

Two theses were defended in the Microbiological Institute of the USSR Academy of Sciences on April 28, 1960.

In the competition for the candidate's degree in the biological sciences A. F. Tranina defended a thesis on the subject "On the interrelationships of certain plants with root microflora."

It was established that the quantity and species composition of the bacteria which predominate and which are constantly present on the roots of wheat differ from those on potatoes. For example, Pseudomonas radiobacter, P. herbicola, Chromobacterium denitrificans are regularly present on the roots of wheat plants but are not encountered at all on the potato. Bacillus mesentericus niger, P. annulata, Mycobacterium album and others which are found on potato roots are never regularly present in any number or prevalence in wheat microflora. The specific agents of bacteriosis in wheat, P. atrofaciens and P. translucens, are never discovered in washings from potato roots and, on the other hand, the potato pathogens P. xanthochlora and Bact. phytophthorum are not in the microfloral composition of the wheat root zone. However, there were instances in which root bacteria were common to the plants being investigated. For example, P. fluorescens M, Bac. mesentericus vulgatus, P. solanacearum and others were encountered on the roots of both wheat and potato.

The quantity of microflora was shown to depend on the method of soil cultivation. Depending on the soil cultivation, a change in moisture regime influences the physiological state of the plant, which determines the basic condition for the existence of the root microorganisms. Some correlated dependence between the soil cultivation method and the quantity of microorganisms on the plant roots in the determinate phase of plant development and at harvest was observed. Thus deep unturned furrow cultivation of the soil always corresponded to the maximum number of bacteria on wheat roots in the earing phase and to the greatest grain harvest.

The species composition of the root microflora did not change with the different types of cultivation.

Bacteria pathogenic to the plants were constantly present on wheat and potato roots. The finding of these on the surface of healthy plant roots was not accompanied by disease of the latter (on wheat roots, P. translucens and P. atrofaciens; on potato roots and tubers, P. solanacearum, P. xanthochlora, P. phytophthorum and others); the vital activity of these bacteria did not depend on the method of cultivation.

Among the phytopathogenic bacteria discovered on wheat roots, the regular presence of P. solanacearum was reported for the first time to stimulate the withering of the Solanaceae and other plants. It was not pathogenic for cereal grain culture. The preservation of virulence in those strains of P. solanacearum which were isolated from wheat roots was experimentally confirmed for affected plants.

Determination of the vigor of the processes of plant tissue maceration effected by the root microflora of wheat and potato under various methods of soil cultivation showed that deep plowing promoted the development of the greatest activity by aerobic cellulose- and pectin-decomposing root microflora. The observations of the decomposition of wheat roots after reaping the harvest, accompanied by histological study of their anatomical structure, confirmed such characteristics of the effect of cultivation methods on the degree of maceration of the root system. Therefore deep plowing is considered by the author as a prophylactic measure which promotes disinfection of the soil, since maceration of plant remains is one of the most important conditions for freeing the soil from the agents of many plant diseases.

The material in this thesis is offered for use on a theoretical basis for possible adoption of deep plowing of the soil in the conditions of the Leningrad region.

### II

In the competition for the candidate's degree in the biological sciences, Yu. V. Kruglov defended a thesis on the subject "Concerning the role of denitrifying bacteria of the genus Pseudomonas fluorescens in the root nutrition of plants."

It was established that P. melochlora, under conditions of hydroponic monobacterial culture of corn, has a significant effect on the growth and nitrogen metabolism of the plant. The nature of this activity in great degree depends on the form of the nitrogen. In Pryanishnikov medium with ammonium nitrate the microorganisms stimulate plant growth and the biosynthesis of proteins, whereas in Knop medium these processes are suppressed.

Under conditions of pure culture in Gil'taya medium P. melochlora synthesizes thiamine, pantothenic acid, pyridoxine and asparagine. The biosynthesis of these, with the exception of thiamine, goes on more vigorous-

*Article not included in Microbiology, Vol. 29, No. 5 (translation published March-April, 1961).

in a medium containing ammonium nitrate. Apparently the ammonium ion present in the medium stimulates these processes.

An analogous situation is observed in the hydroponic culture of corn. The microorganisms, using the energy-supplying material secreted by the roots into the Pryanishnikov medium, form pyridoxine, which accumulates in the solution and is taken up by the plant. In Knop medium these processes are negligible.

Comparing the material obtained by microbial biosynthesis of the biological substances with data from vegetative experiments, the author comes to the conclusion that the capacity of these microorganisms to actively form vitamins and especially pyridoxine, which plays an exceptionally large role in the process of nitrogen metabolism, is one of the basic factors of the positive action of P. melochlora on the root system of plants.

A certain quantity of asparagine added to the basic mineral nutrients is shown to stimulate plant growth. So long as the concentration of nitrogen (including asparagine) does not exceed 4-5 mg in 2 liters of medium the author considers that this nitrogen cannot play a substantial role in plant nutrition. Evidently in the given instance its role is limited by the catalysis of some processes in the plant organism.

The denitrifying bacteria investigated readily ammonify peptone, aspartic acid, alanine, and glutamic acid, using them as carbon sources and hydrogen donors in the processes of nitrate reduction. The only exception was peptone; reduction of nitrates was not observed in its presence.

It was established that in vegetative experiments in medium with peptone as the single nitrogen source, P. melochlora stimulates plant growth. In these instances the bacteria ammonify peptone, supplying the plant root system with an accessible form of nitrogen.

III

The defense of two theses took place in the Microbiological Institute, Academy of Sciences, USSR, on June 15, 1960.

In the competition for the doctor's degree in the biological sciences, E. I. Kvasnikov defended a thesis on the subject "The biology of the lactic acid bacteria."

The general characteristics of the group of lactic acid bacteria were presented.

It was shown that one of the most characteristic capacities of the lactic acid bacteria is their high alcohol stability. Media containing alcohol were suggested as selective for the cultivation of the bacteria being studied. New methods of isolation and quantitative assay of lactic acid bacteria in different substrates containing a large quantity of extraneous microorganisms were devised on the basis of using these media.

Investigations carried out with the acid of such methods permit a new treatment of the ecology of these microorganisms and show that the main habitat of many of these specimens is in the soil, where they are concentrated in the rhizospheres of plants. The lactic acid bacteria are among the most widespread groups of microorganisms in nature. In natural and standard industrial substrates they most often develop in close interdependence with those organisms which possess the highest auxoautotrophism in comparison with themselves. This is precisely how the lactic acid bacteria fulfill their great needs for vitamins and amino acids.

These substances occur in the rhizospheres of plants (in the root secretions and products of rhizosphere metabolism of the auxoautotrophic microorganisms), in the intestinal tract of animals (apparently also mainly because of the microorganisms inhabiting it), in a number of industrial sugar-containing substrates (mainly in the presence of yeasts, developing in association with them).

The author proposes that the great auxoheterotrophism of many lactic acid bacteria has been formed in a process of long evolution in close contact with these representatives of the organic world.

On the basis of knowing the special biology of the lactic acid bacteria the principles of regulating their vital activity in several branches of the national economy have been worked out.

New races of microorganisms, new methods of investigation and microbiological control having been elucidated, a recommended system of measures for the regulation of the vital activity of the lactic acid bacteria in standard industrial substrates has found application in practice.

IV

In the competition for the candidate's degree in the biological sciences, E. G. Popova defended a thesis on the topic "Microflora of the takyrs in connection with the problem of their exploitation."

It was established that the lichen-algal takyrs of the Prikopetdag burned zone in the natural condition are characterized by inferior biogeny. The best development of microorganisms was observed in the superficial genetic soil horizons. In the horizon from 6-28 cm a significant decrease in the numbers of microbes was noted. Deeper than 28 cm the numbers fell sharply. The distribution of microorganisms in the vertical profile of a cross-section of the soil shows that the processes of soil formation in the takyr affect strata no deeper than 28-30 cm.

In the process of the formation of takyrs it was established that all groups of soil microorganisms took part. Bacteria, actinomycetes and algae all had basic importance in the quantitative complement of microbes. In view of their insignificant concentration, fungi occupy a minor position in the biocenosis of the lichen-algal takyrs. However, some of these species take part in the decomposition of cellulose. In the composition of the bacterial population the nonsporulating species predominate. Nitrifying bacteria in small numbers are found in the superficial layers, to a depth of 28 cm. Azotobacter was not recorded by the methods utilized.

Among the fungi in the takyr crust, representatives of the genera Macrosporium and Stemphylium predominated. At lower-lying levels those species of

fungus which bear sterile mycelia were dominant. The chief role in the decomposition of cellulose belongs to the actinomycetes and fungi.

Takyr-type soil, in contrast to takyrs, is characterized by much higher biogeny. This soil occupies an intermediate position between the takyr and serozem in regard to its content of major groups of microorganisms. In takyr-like soils azotobacter were found. Bac. mesentericus and Bac. Idosus were the dominant bacterial among sporulating forms. Representatives of the genera Penicillium and Aspergillus were the leading roups of fungi. In the decomposition of cellulose the chief roles were played by actinomycetes and fungi.

Serozem is characterized by a high content of aerobic and anaerobic bacteria, actinomycetes and cellulose-decomposing microorganisms. Azotobacter chroococcum is found in serozem. The prevalent groups of sporulating bacteria are Bac. mesentericus and Bac. idosus. Representatives of the genus Aspergillus dominate the fungal flora.

In the development of a microflora in virgin lands of the Prikopetdag burned zone a seasonal prevalence is observed. The greatest number of different groups of microorganisms is found in the spring, autumn, and winter, that is, in the periods which are favorable to their development thanks to the presence of warmth and moisture in the soil. In the summer and beginning of autumn the vital activity of the microbes declines; however, several of them possess great resistance to a dry environment and retain their viability.

Irrigation of these soils and agricultural cultivation is bound to promote the activation of microbiological processes and the increase of soil fertility.

In connection with the negligible content of organic substances in the takyr and the high salinity of this soil for the intensification of microfloral activity and the increase of soil fertility, irrigation by itself is not sufficient. A radical improvement in soil properties, leaching our of harmful salts, the introduction of organic fertilizer and the sowing of crops are necessary.

As the result of cultivation of the lichen-algal takyrs a sharp increase in the biological activity of the soil has been attained. Greater numbers of microbes have been revealed in the data from experiments with preliminary leaching of salts from the soil. In comparison with the virgin soil the content of bacteria, actinomycetes, and fungi in the cultivated takyr increased 10-35 times in ploughed sections. In ploughed-under horizons of the cultivated takyr the usual number of bacteria is 90-350 times greater than in the virgin land, the number of actinomycetes is increased 40-150 times, and the number of molds is increased 50-90 times.

Cultivation showed an especially favorable effect on the enrichment of the takyrs by nitrifying, cellulose-decomposing bacteria and azotobacter. In ploughed areas of the cultivated takyr the numbers of nitrifying bacteria and of cellulose-decomposing bacteria were, respectively, 100 and 50-200 times greater than in the virgin soil. Azotobacter was found at a depth of 75 cm. A decrease in the number of forms with sterile mycelia and an increase in the number of Mucor-type forms and representatives of the genera Fusarium and Trichoderma were observed among the microscopic fungi in the soil of the cultivated takyr.

## V

Two theses were defended on May 20, 1960 in Kiev before the Joint Academy Council of the Section on Biological Sciences, Academy of Sciences, USSR.

In the competition for the doctor's degree in the biological sciences Kh. G. Zinov'eva defended a thesis on the subject "Interrelationships of azotobacter with higher plants."

Study of the variability of azotobacter in the process of its symbiotic development with a number of agricultural crops (wheat, oats, corn, potatoes, sugar beets) showed that under the influence of the plants important changes in its cultural, morphological and physiological properties took place. Separate strains of azotobacter reacted differently to the influence of the root system of the mature plant. In one strain the economically useful properties were increased but in another, on the other hand, they were lost. In further joint cultivation with suitable plants several transformed variants of azotobacter preserved the changes acquired by them, others returned to their original forms and in some a decrease in nitrogen fixation and acclimatization to the roots was observed. Some azotobacter variants obtained by the influence of the plants possessed a higher decomposition and nitrogen-fixing activity, better acclimatization to the rhizosphere of the appropriate plant and in great degree increased the harvest in comparison to the corresponding original strain or to cultures adapted to other plants. The azotobacter variants which were changed by their relationship to various plants reacted differently to the root secretions of one and the same plant; in the presence of root secretions from unsuitable plants their decomposing activity and their root acclimatization decreased in one way or another. In the presence of other sources of nutrients in the medium, the adapted cultures of azotobacter primarily used the substances secreted from the roots of their particularly suited plants. They manifested unequal treatment of different carbon sources and showed considerable differences in fermentative activity. Thus, for example, the catalase activity in two-, five-, and ten-day cultures was much higher than in the original strains. These increases occurred also for amylase, dehydrase and urease activities. The altered cultures of azotobacter showed a positive effect on the availability of soluble forms of nitrogen (nitrates and ammonia) in the rhizospheres and on the increase in the usual number of bacteria growing on MPA and starch-ammoniac agar. In some variants the changes acquired in the interaction with the plants were incorporated hereditarily. Adaptational variants showed a positive effect not only on suitable plants but also on other types of agricultural crops and increased

their harvest, however. in lesser degree than the harvest of their appropriate plants.

The active variant of azotobacter (No. 28) was obtained by means of cultivation in wheat rhizospheres. In 1957 it was approved by the All-Union Agricultural Microbiology Scientific-Research Institute and was recommended for productive application in the conditions of the Ukrainian SSR.

In the manufacture of azotobacterin, variant 28 manifested a much greater decomposing activity than the standard strains K and 53 and maintained a heightened nitrogen-fixing activity. Azotobacterin prepared in variant 28 increased the germination energy of wheat seeds and the harvest of this plant in greater degree than the azotobacterin prepared in strain K. After four years of storage in laboratory conditions variant 28 retains its acquired characteristics of better acclimatization in the wheat rhizosphere, a greater nitrogen-fixing activity and greater effective action on the harvest in comparison with the original strain K.

## VI

In the competition for the doctor's degree in the biological sciences L. Yu. Medvedinskaya defended a thesis on the subject "Bacteriophage of lactic acid streptococci."

The distribution of lactic acid streptococcal bacteriophage in cheese and butter manufacturing plants in the Ukrainian SSR was established. It was shown that it provokes a disturbance or cessation of the lactic acid process in the manufacture. A hundred phages which lyse cultures of Streptococcus lactis, S. cremoris, S. citrovorus and S. paracitrovorus were isolated. Phages of two of these cultures were isolated for the first time.

In tests of 79 phages on 180 cultures of lactic acid streptococci, seven types of phage were established for S. lactis. Two of these, the most widespread in the manufacturing plants, lysed the majority of tested cultures (158 out of 180). They were designated as phage Types I and II. The remaining five S. lactis phage types lysed nine cultures in all. Cross-tests of each type of S. lactis phage on cultures which were lysed by the remaining phage types showed their type specificity. S. lactis phage Types I and II are distinguished by morphology, by the character of their plaques, single step curve, serological and other properties. S. lactis Type I phage lysed not only cultures of S. lactis but also S. cremoris. Type I phage succeeded in adapting to the lysis of S. diacetilactis cultures and phage was obtained from this experiment which had new properties, giving plaques of altered shape. The new properties of the phage were stably transferred to its descendants over many passages. The serological peculiarities of S. lactis cultures which were lysed by the two main phage types were studied. The connection between the serological properties of the cultures and the types of phage which lyse them was established. It appeared that the species S. lactis is serologically heterogeneous. The variability of S. lactis cultures under the action of Types I and II S. lactis phage and also the variability of S. cremoris when affected by homologous phages were studied.

When acted upon by a high concentration of Type I S. lactis and S. cremoris phages, the population of cultures of S. lactis and S. cremoris consisting of cells heterogeneous for sensitivity formed three groups of secondarily resistant cultures. When acted upon by higher concentrations of Type II S. lactis phage the populations of S. lactis cultures consisting almost entirely of phage-sensitive cells form only one group of secondarily phage-resistant cultures, similar to the first group of secondary cultures which was obtained by the action of a high concentration of Type I phage. In the first group of secondary cultures of S. lactis obtained by the action of a high concentration of Types I and II, cultural, morphological, biochemical and serological properties were sharply altered. They became phage-resistant. The changed characteristics were stably transmitted to descendants for many generations. The secondary, phage-resistant cultures belonging to the first group are so clearly distinguished from the original cultures of S. lactis as to exceed species bounds. Secondary, phage-resistant cultures of S. lactis belonging to the second and third groups obtained by the action of greater and lesser concentration of Type I S. lactis phage and lesser concentration of Type II phage represent different variants within this species.

It was established that a complicated interrelationship exists between specific phage and cultures of lactic acid streptococci. The characteristics of secondary cultures are conditioned by this relationship. It depends on the phage type, the activity and concentrantion of the phage, the properties of the original culture, the number of phage-sensitive cells in the population, and the degree of phage sensitivity.

For the first time filterable forms of S. lactis were obtained from old cultures and also by the action of bacteriophage, by freezing and thawing and by mechanical disruption of the cells. The filterable forms of S. lactis were discovered also in milk and milk products. Cultures regenerated from filterable forms had heterogeneous characteristics which bore witness to the qualitative differences in filterable forms of S. lactis. The author contends that the filterable forms are cells adapted to unfavorable conditions in the medium. The filterable forms are not developmental stages in the ontogenesis of bacterial cells.

The work carried out on the investigation of S. lactis phage types widespread among cheese and butter manufacturing plants in the Ukrainian SSR, data on the phage typing of S. lactis cultures and their variability has given us the possibility to apply these principles to the selection of S. lactis and S. cremoris cultures for fermentation.

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## RUSSIAN JOURNALS FREQUENTLY CITED
### [Biological Sciences]

| Abbreviation* | Journal* | Translation |
|---|---|---|
| Agrobiol. | Agrobiologiya | Agrobiology |
| Akusherstvo i Ginekol. | Akusherstvo i Ginekologiya | Obstetrics and Gynecology |
| Antibiotiki | Antibiotiki | Antibiotics |
| Aptechnoe Delo | Aptechnoe Delo | Pharmaceutical Transactions |
| Arkh. Anat. Gistol. i Émbriol. | Arkhiv Anatomii Gistologii i Émbriologii | Archives of Antomy, Histology, and Embryology |
| Arkh. Biol. Nauk SSSR | Arkhiv Biologicheskikh Nauk SSSR | Archives of Biological Science USSR |
| Arkh. Patol. | Arkhiv Patologii | Archives of Pathology |
| Biofizika | Biofizika | Biophysics |
| Biokhimiya | Biokhimiya | Biochemistry |
| Biokhim. Plodov i Ovoshchei | Biokhimiya Plodov i Ovoshchei | Biochemistry of Fruits and Vegetables |
| Bot. Zhur. | Botanicheskii Zhurnal | Journal of Botany |
| Byull. Éksptl. Biol. i Med. | Byulleten Éksperimentalnoi Biologii i Meditsiny | Bulletin of Experimental Biology and Medicine |
| Byull. Moskov. Obshchestva Ispytatelei Prirody, Otdel Biol. | Byulleten Moskovskogo Obshchestva Ispytatelei Prirody, Otdel Biologicheskii | Bulletin of the Moscow Naturalists Society, Division of Biology |
| Doklady Akad. Nauk SSSR | Doklady Akademii Nauk SSSR | Proceedings of the Academy of Sciences USSR |
| Éksptl. Khirurg. | Éksperimentalnaya Khirurgiya | Experimental Surgery |
| Farmakol. i Toksikol. | Farmakologiya i Toksikologiya | Pharmacology and Toxicology |
| Farmatsiya | Farmatsiya | Pharmacy |
| Fiziol. Rastenii | Fiziologiya Rastenii | Plant Physiology |
| Fiziol. Zhur. SSSR | Fiziologicheskii Zhurnal SSSR im. I. M. Sechenova | I. M. Sechenov Physiology Journal USSR |
| Gigiena i Sanit. | Gigiena i Sanitariya | Hygiene and Sanitation |
| Izvest. Akad. Nauk SSSR, Ser. Biol. | Izvestiya Akademii Nauk SSSR, Seriya Biologicheskaya | Bulletin of the Academy of Sciences USSR, Biology Series |
| Izvest. Tikhookeanskogo N. I. Inst. Rybnogo Khoz. i Okeanog. | Investiya Tikhookeanskogo N. I. Instituta Rybnogo Khozyaistva i Okeanografii | Bulletin of the Pacific Ocean Scientific Institute of Fisheries and Oceanography |
| Khirurgiya | Khirurgiya | Surgery |
| Klin. Med. | Klinicheskaya Meditsina | Clinical Medicine |
| Lab. Delo | Laboratornoe Delo (po Voprosam Meditsiny) | Laboratory Work (on Medical Problems) |
| Med. Parazitol. | Meditsinskaya Parazitologiya i Parazitarnye Bolezni | Medical Parasitology and Parasitic Diseases |
| Med. Radiol. | Meditsinskaya Radiologiya | Medical Radiology |
| Med. Zhur. Ukrain. | Medichnii Zhurnal Ukrainskii | Ukrainian Medical Journal |
| Mikrobiologiya | Mikrobiologiya | Microbiology |
| Mikrobiol. Zhur. | Mikrobiologichnii Zhurnal | Microbiology Journal |
| Nevropatol., Psikhyat. i Psikhogig. | Nevropatologiya, Psikhyatriya i Psikhogigiena | Neuropathology, Psychiatry and Psychohygiene |
| Ortoped., Travmatol. i Protez. | Ortopediya, Travmatologiya i Protezirovanie | Orthopedics, Traumatology and Prosthetics |
| Parazitol. Sbornik | Parazitologicheskii Sbornik | Parasitology Collection |
| Pediatriya | Pediatriya | Pediatrics |
| Pochvovedenie | Pochvovedenie | Soil Science |
| Priroda | Priroda | Nature |
| Problemy Éndokrinol. i Gormonoterap. | Problemy Endokrinologii i Gormonoterapii | Problems of Endocrinology and Hormone Therapy |
| Problemy Gematol. | Problemy Gematologii i Perelivaniya Krovi | Problems of Hematology and Blood Transfusion |
| Problemy Tuberk. | Problemy Tuberkuleza | Problems of Tuberculosis |
| Sovet. Med. | Sovetskaya Meditsina | Soviet Medicine |
| Sovet. Vrachebny Zhur. | Sovetskii Vrachebnyi Zhurnal | Soviet Physicians Journal |
| Stomatologiya | Stomatologiya | Stomatology |

*BRITISH-AMERICAN TRANSLITERATION SYSTEM.

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| Abbreviation | Journal | Translation |
|---|---|---|
| Terap. Arkh. | Terapevticheskii Arkhiv | Therapeutic Archives |
| Trudy Gelmint. Lab. | Trudy Gelmintologicheskoi Laboratoriya | Transactions of the Helminthology Laboratory |
| Trudy Inst. Genet. | Trudy Instituta Genetiki | Transactions of the Institute of Genetics |
| Trudy Inst. Gidrobiol. | Trudy Instituta Gidrobiologiya | Transactions of the Institute of Hydrobiology |
| Trudy Inst. Mikrobiol. | Trudy Instituta Mikrobiologiya | Transactions of the Institute of Microbiology |
| Trudy Inst. Okean. | Trudy Instituta Okeanologiya, Akademii Nauk SSSR | Transactions of the Institute of Oceanology, Academy of Sciences, USSR |
| Trudy Leningrad Obshchestva Estestvoisp. | Trudy Leningrad Obshchestva Estestvoispytatelei | Transactions of the Leningrad Society of Naturalists |
| Trudy Vsesoyuz. Gidrobiol. Obshchestva | Trudy Vsesoyuznogo Gidrobiologicheskogo Obshchestva | Transactions of the All-Union Hydrobiological Society |
| Trudy Vsesoyuz. Inst. Eksptl. Med. | Trudy Vsesoyuznogo Instituta Eksperimentalnoi Meditsiny | Transactions of the All-Union Institute of Experimental Medicine |
| Ukrain. Biokhim. Zhur. | Ukrainskii Biokhimichnii Zhurnal | Ukrainian Biochemical Journal |
| Urologiya | Urologiya | Urology |
| Uspekhi Biokhimiya | Uspekhi Biokhimiya | Progress in Biochemistry |
| Uspekhi Sovremennoi Biol. | Uspekhi Sovremennoi Biologiya | Progress in Contemporary Biology |
| Vestnik Akad. Med. Nauk SSSR | Vestnik Akademii Meditsinskikh Nauk SSSR | Bulletin of the Academy of Medical Science USSR |
| Vestnik Khirurg. im. Grekova | Vestnik Khirurgii imeni Grekova | Grekov Bulletin of Surgery |
| Vestnik Leningrad. Univ. Ser. Biol. | Vestnik Leningradskogo Universiteta, Seriya Biologii | Journal of the Leningrad Univ., Biology Series |
| Vestnik Moskov. Univ., Ser. Biol. i Pochvov. | Vestnik Moskovskogo Universitera, Seriya Biologii i Pochvovedeniya | Bulletin of the Moscow University, Biology and Soil Science Series |
| Vestnik Oftalmol. | Vestnik Oftalmologii | Bulletin of Ophthalmology |
| Vestnik Oto-rino-laringol. | Vestnik Oto-rino-laringologiya | Bulletin of Otorhinolaryngology |
| Vestnik Rentgenol. i Radiol. | Vestnik Rentgenologii i Radiologii | Bulletin of Roentgenology and Radiology |
| Vestnik Venerol. i Dermatol. | Vestnik Venerologii i Dermatologii | Bulletin of Venereology and Dermatology |
| Veterinariya | Veterinariya | Veterinary Science |
| Vinodelie i Vinogradarstvo | Vinodelie i Vinogradarstvo SSSR | Wine-Making and Viticulture |
| Voprosy Klin. | Voprosy Klinicheskie | Clinical Problems |
| Voprosy Med. Khim. | Voprosy Meditsinskoi Khimii | Problems of Medical Chemistry |
| Voprosy Med. Virusol. | Voprosy Meditsinskoi Virusologii | Problems of Medical Virology |
| Voprosy Neirokhirurg. | Voprosy Neirokhirugii | Problems of Neurosurgery |
| Voprosy Onkol. | Voprosy Onkologii | Problems of Oncology |
| Voprosy Pitaniya | Voprosy Pitaniya | Problems of Nutrition |
| Voprosy Psikhologii | Voprosy Psikhologii | Problems of Psychology |
| Voprosy Virusologii | Voprosy Virusologii | Problems of Virology |
| Vrachebnoe Delo | Vrachebnoe Delo | Medical Profession |
| Zav. Lab. | Zavodskaya Laboratoriya | Factory Laboratory |
| Zhur. Mikrobiol., Epidemiol. i Immunobiol. | Zhurnal Mikrobiologii, Epidemiologii i Immunobiologii | Journal of Microbiology, Epidemiology, and Immunobiology |
| Zhur. Nevropatol. i Psikhiat. | Zhurnal Nevropatologii i Psikhiatrii imeni S. S. Korsakov | S. S. Korsakov Journal of Neuropathology and Psychiatry |
| Zhur. Obshchei Biol. | Zhurnal Obshchei Biologiya | Journal of General Biology |
| Zhur. Vysshei Nerv. Deyatel. | Zhurnal Vysshei Nervnoi Deyatelnosti imeni I. P. Pavlova | I. P. Pavlov Journal of Higher Nervous Activity |
| Zool. Zhur. | Zoologicheskii Zhurnal | Journal of Zoology |

## ABBREVIATIONS MOST FREQUENTLY ENCOUNTERED IN RUSSIAN BIO-SCIENCES LITERATURE

| Abbreviation (Transliterated) | Significance |
|---|---|
| AMN SSSR | Academy of Medical Sciences, USSR |
| AN SSSR | Academy of Sciences, USSR |
| BIN | Biological Institute, Botanical Institute |
| FTI | Institute of Physiotherapy |
| GONTI | State United Sci-Tech Press |
| GOST | All Union State Standard |
| GRRRI | State Roentgenology, Radiology, and Cancer Institute |
| GTTI | State Technical and Theoretical Literature Press |
| GU | State University |
| I Kh N | Scientific Research Institute of Surgical Neuropathology |
| IL (IIL) | Foreign Literature Press |
| IONKh | Inst. Gen. and Inorganic Chemistry (N. S. Kurnakov) |
| IP | Soil Science Inst. (Acad. Sci. USSR) |
| ISN (Izd. Sov. Nauk) | Soviet Science Press |
| Izd. | Press |
| LEM | Laboratory for experimental morphogenesis |
| LENDVI | Leningrad Inst. of Dermatology and Venereology |
| LEO | Laboratory of Experimental Zoology |
| LIKhT | Leningrad Surgical Institute for Tuberculosis and Bone and Joint Diseases |
| LIPZ | Leningrad Inst. for Study of Occupational Diseases |
| LIPK | Leningrad Blood Transfusion Institute |
| Medgiz | State Medical Literature Press |
| MOPISh | Moscow Society of Apiculture and Sericulture |
| MVI | Moscow Veterinary Institute |
| MZdrav | Ministry of Health |
| MZI | Moscow Zootechnical Institute |
| LOKhO | Leningrad Society of Orthopedic Surgeons |
| NIIZ | Scientific Research Institute of Zoology |
| NINKhI | Scientific Research Institute of Neurosurgery |
| NIU | Scientific Institute for Fertilizers |
| NIUIF | Scientific Research Institute of Fertilizers and Insecticides |
| NIVI | Veterinary Scientific Research Institute |
| ONTI | United Sci. Tech. Press |
| OTI | Division of Technical Information |
| RBO | Russian Botanical Society |
| ROP | Russian Society of Pathologists |
| SANIIRI | Central Asia Scientific Research Institute of Irrigation |
| SANIISh | Central Asia Scientific Research Institute of Sericulture |
| TsNII | All-Union Central Scientific Research Institute |
| TsNTL | Central Scientific and Technical Laboratory |
| VASKhNIL | All-Union Academy of Agricultural Sciences |
| VIG | All-Union Institute of Helminthology |
| VIEM | All-Union Institute of Experimental Medicine |
| VIR | All-Union Institute of Plant Cultivation |
| VIUAA | All-Union Institute of Fertilizers, Soil Science, and Agricultural Engineering |
| VIZR | All-Union Institute of Medical and Pharmaceutical Herbs |
| VNIRO | All-Union Scientific Institute of Fishing and Oceanography |
| ZIN | Zoological Inst. (Acad. Sci. USSR) |

Note: Abbreviations not on this list and not explained in the translation have been transliterated, no further information about their significance being available to us. - Publisher.

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